1967

Studies of the outer wall of epidermal cells of leaves

Charles Grieve Waywell

Iowa State University

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Iowa State University of Science and Technology, Ph.D., 1967
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STUDIES OF THE OUTER WALL
OF EPIDERMAL CELLS OF LEAVES

by

Charles Grieve Waywell

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Plant Physiology

Approved:
Signature was redacted for privacy.

In Charge of Major Work
Signature was redacted for privacy.

Head of Major Department
Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

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INTRODUCTION

Men have been curious about the function of leaves for many years. Stephan Hales, (49) in the early part of the eighteenth century, conducted numerous experiments on the growth of leaves and of their function. Approximately a century later Brongniart (11, 12) reported on the structure and function of leaves and was one of the first to realize the importance of the cuticle. Ninety years ago deBary (28) described the waxy covering of leaves and published drawings showing not only details of leaf epidermal cell structure but the shape and distribution of wax particles and 'rodlets.'

Early investigators were primarily interested in structural phenomena which prevented desiccation of leaves. Studies of the factors affecting water loss or retention have been mainly concerned with the waxy coverings of leaves; with the thickness, extent and nature of the cuticle; and with the location, size, number, and distribution of the stomatal openings.

In recent years the main emphasis has been on the way in which various materials are absorbed through the leaf surface. The difference in response of plant species to herbicides, changes in response to herbicides over the growing season, increased interest in the possible use of systemic insecticides and fungicides, and the growth of the practice of applying trace elements and other nutrients to crops as foliar applica-
tions has made investigators aware of the need for a more de­
tailed knowledge of the structure of the leaf epidermis.

This study was made to learn more about the wax layer on
the surface of the leaf, about the structure of the cuticle
on different species, and on the fine structure of the outer
wall of the epidermal cells of the leaf. The rapid develop­
ment of new techniques for use with the electron microscope
has made it possible to observe detail which is beyond the re­
solving power of the light microscope.

The present study has therefore been devoted to these
projects:

1. A comparison of the polyvinyl alcohol cast and
   the carbon replica techniques in the study of
   the wax layer.

2. A comparison of the results of these studies
   with the work reported by deBary and other rep­
   resentatives of the classical school of light
   microscopists.

3. A study of the external and internal surfaces of
   the cuticle.

4. A study of sections through the cuticle to deter­
   mine its structure and the possible relation of
   this structure to the formation of the wax layer,
   and

5. A study of ultra thin sections through the outer
   wall of the leaf epidermal cell to determine the
relationship of its structure with the formation of the cuticle and wax layer.

6. A study of sections through young epidermal cells to determine the relationship between the outer cell wall and the protoplast.
REVIEW OF LITERATURE

Historical

Centuries ago gardeners, herbalists and other men who spent much of their time with plants probably had ideas concerning the importance of leaves in the scheme of plant growth. However, it was not until the invention of the microscope that men were able to study the finer structures of plants. Marcello Malphigi (76) discovered the stomata or "breathing-pores" of leaves and it has been assumed that he understood their function. Nehemiah Grew (48) a contemporary of Malphigi, wrote that "a Plant lives partly upon Aer, for the reception whereof, it hath those Parts which are answerable to Lungs." Stephen Hales (49) in his book Vegetable Staticks states "that one great use of leaves is what has been long suspected by many, viz, to perform in some measure the same office for the support of the vegetable life, that the lungs of animals do, for the support of animal life; Plants very probably drawing through their leaves some part of their nourishment from the air."

Hales placed branches cut from several kinds of trees in separate glasses and poured into each glass known quantities of water. The leaves were stripped from one bough of each sort. After twelve hours the leafless bough had imbibed one ounce of water while the others had imbibed fifteen to thirty ounces, more or less in proportion to the quantity of leaves they had.
Hales not only performed a number of experiments which proved that leafy plants "perspired" but also by immersing branches and leaves in water measured the quantity of water absorbed through the leaves. This he found to be much less than could be lost by the same branch when exposed to air. In Experiment VIII (p. 17) Hales measured the amount of water "imbibed and perspired" by (1) an apple stem with a large apple and twelve leaves, (2) a similar stem with no apple but the same leaf area, and (3) a third stem with two large apples on it but without leaves. His conclusion was "therefore two leaves imbibe and perspire as much as one apple; whence their perspirations seem to be proportional to their surfaces; the surface of the apple being nearly equal to the sum of the upper and under surface of the two leaves." This was the earliest statement found relating the function of leaves to a surface phenomena.

The experiments of Joseph Priestley (105) demonstrated that plants had the reverse affect on air as did the respiration of animals. Jan Ingenhousz (58) reported that "green leaves purify the air when in sunlight but at night, they act like animals." In 1804 de Saussure (29) published the results of his experiments which showed that green plants exposed to light absorbed carbon dioxide in equal volume to the oxygen they exhaled.

Many other investigators were active in the study of respiration but it was not until Brongniart (11, 12) that there
was much research reported on the relationship of the structure of leaves to their function. His first paper was devoted to a detailed description of the leaf structure. At that time some authors considered that the epidermis consisted of a simple membrane without any appreciable structure, others believed it to be the exterior surface or outer walls of cells, while others considered the epidermis to be a layer of cells distinct from the parenchyma cells beneath. Brongniart studied many cross sections of leaves under the microscope to determine which hypothesis was true. He concluded that the epidermis was not a simple membrane but a layer of cells adhering to each other, but only lightly to the cells of the mesophyll layer beneath. He reported that the cells of the mature epidermis were transparent, colorless, and that the walls, particularly the outer wall, were thicker than those of the cells beneath.

By soaking parts of cabbage leaves in water for long periods of time he was able to isolate a simple membrane which was transparent and non-cellular. This simple membrane, he claimed, covered the outer walls of the epidermal cells. Further work on many other plants (12) convinced Brongniart that this non-cellular covering was generally present in both monocots and dicots.

Brongniart also removed the epidermis of some leaves and proved that the epidermis protected the leaf against too rapid evaporation. He also demonstrated that the intercellular
spaces in the spongy mesophyll layers of leaves were continuous and that "communication with the outside air was possible through the stomata. --- One can see, therefore, that there is an analogy greater than one would have been able to expect between the respiration of plants in air and that of animals by means of lungs --- the cells which carry on respiration are separated from the air by a very thin moist membrane --- and these surfaces are prevented from drying out by too rapid renewal of exterior air."

DeBary (28) in 1871 described the structure of the waxy covering of leaves. Figures 13 to 16 are reproductions of portions of the plates from his paper. He described several types of wax; a flour-like powder, crustose layers, granular, and rod-like forms. This wax "comes out unchanged through the growing membrane and cuticle. --- How it comes through is another question and I will not discuss this problem." DeBary agreed with those who thought that these secretions were the product of growing cellulose membranes and cuticle. --- "It is clear that wax substances are developed from carbohydrates --- that they are a reduction product of carbohydrates."

Importance of the Leaf Surface

Although the basic structure of the epidermis has been known for almost a century there are many unanswered questions which have engaged the attention of research workers over the
last thirty years. Most of these questions have been concerned with how absorption or penetration takes place through the epidermal layer. Fogg (36), Staniforth and Loomis (131), Schieferstein and Loomis (121), and Linskens (74) have been concerned with the factors affecting the ability to wet the leaf surface. Roberts, et al. (109), Orgell (95), and Currier and Dybing (25) have studied the penetration of the leaf surface by spray residues. Roberts and Martin (110), Brown and Harvey (13), and Johnson (60) have presented the results of their studies on the method by which fungal hyphae effect penetration. McAllan and Adams (79) were concerned with the structure of the epidermis and how it could be penetrated by aphids. Bukovac and Wittwer (14) and Boynton (8) reported on the absorption of nutrients through the leaf surface, while van Overbeek (140) presented a review of the absorption and subsequent translocation of growth regulators. This brief resume indicates that the epidermal layer is of fundamental importance to plant physiologists, plant pathologists, weed technologists, and entomologists.

The Wax Layer

Physical structure

Throughout the literature concerning the wax layer references are made to deBary (28) and his extensive report on the subject which appeared in 1871. In his paper, however, deBary writes as though the presence of a layer of wax had
been generally accepted by botanists for some years. He recognized that the color and form of the wax particles or layers varied from one species to another. He also stated that on different parts of plants there were several kinds of wax which varied in chemical composition. The results of his investigations convinced him that the wax layer was comprised of a mixture and that the physical characteristics of the wax layer were determined by the varied proportions of the different waxes. In a number of his illustrations he shows rod-like structures which he considered to be characteristic for certain species of plants. Three other types of wax were also described; as lumps evenly spread over the leaf, as simple particles or grains, and in the form of thin layers with no definite structure.

Wiesner (150) developed the thesis that all wax layers were crystalline in nature. He stated that a very simple layer of rods or grains cannot be seen clearly even if one is using the greatest magnification. He therefore decided to use a simple chemical test and the polarizing microscope to prove or disprove his contention. To obtain wax he poured warm ether over the surface of cabbage and onion leaves. The liquid was collected in a clean porcelain dish until a sufficient quantity was obtained. The eluate was poured into two test tubes which were then placed in a water bath to evaporate the ether. The precipitated wax in one test tube was treated with water free phosphoric acid and warmed. The
characteristic smell was considered to be proof of the presence of glycerides. From this Wiesner deduced that the surface wax consisted of solid fatty acids and their glycerides which were known to be crystalline. He also used the polarizing microscope in studying the wax of two species of plants and obtained the characteristic bi-refringence produced by crystalline substances. Different solvents were used to remove the wax and it was found that by using benzol that hexagonal crystals, star shaped crystals, and in some cases needle-like crystals were formed when the solvent was evaporated. According to Wiesner "The formed wax layers are no doubt coming out from solution which exudes from the cell walls --- and will develop again and again." The rod-like elements described by deBary were therefore simply crystal aggregates. Weber's results (147) supported Wiesner's conclusions.

Pohl (102) developed a simple method of studying the wax layer. He pressed glass microscope slides against the young leaves of Lupinus albus and Tulipa silvestris and observed small liquid droplets with smooth outlines on the slides. Irregular particles with only a few liquid droplets were found on slides pressed against older leaves. Pohl considered that this proved that younger leaves exuded oil droplets and that mature leaves exuded wax. As Schieferstein (119) reported, this can also be interpreted as an indication that the wax is excreted in solution in a volatile solvent.
Kreger (57) studied wax structure on plant tissue with an X-ray diffraction technique. His conclusions support those of Wiesner in that the distinctive structures are the result of crystallization of the wax on the leaf surface.

Mueller et al. (87) developed a double replica technique to study the leaf surface. Micrographs taken when the replicas were examined in the electron microscope showed characteristic wax patterns. No evidence of liquid wax was found. They concluded that wax was exuded in a softened form.

Schieferstein (119) and Schieferstein and Loomis (121) continued the study initiated by Mueller et al. (87). They found that the material which appeared first on the surface was either a liquid solution of wax or a soft paste. Crystalline wax was apparent later in the development of the leaf.

Juniper and Bradley (63) used a carbon replica technique which they considered superior to the double replica technique used by Mueller and Schieferstein. Juniper (62) assumed that the minute projections seen in the micrographs to be wax extruded through the cuticle. No pores or apertures from which some of the larger projections could be derived were seen and he concludes that they must have been extruded as liquids.

Chemical composition

Channon and Chibnall (17) refluxed air dried cabbage leaves with warm petroleum ether to extract ether soluble substances. After reducing the volume of the extract by distil-
lation, the wax was precipitated by addition of two volumes of acetone. The acetone-soluble fatty substances were considered to be the non-wax fraction. Samples obtained in this way were used in identifying chemically constituents of plant wax. The wax fraction was approximately 0.4 per cent of the total leaf solids. The chief constituents of the wax were monacosane \((C_{29}H_{60})\) and di-n-tetradecyl ketone \((C_{14}H_{29} \cdot CO \cdot C_{14}H_{29})\).

Chibnall and his associates continued this line of research using a number of plant species. The conclusion reached in this work was that cuticle waxes consist of long-chain, even-numbered primary alcohols, acids, and their esters, and of long-chain, odd-numbered secondary alcohols, ketones and paraffin hydrocarbons. Wanless et al. (142) have reported the presence of all the normal paraffins within the range \(C_{24}\) to \(C_{36}\) in Pyrethrum cuticle wax.

Kurtz (68) used a method similar to that of Channon and Chibnall to obtain plant wax from thirteen species indigenous to southern Arizona. Specimens representing seven ages of leaves and nodes were collected. Chemical characterization of the wax fractions was accomplished by determining the acid, saponification, ester, and iodine numbers. These tests give respectively the amount of free acid, saponifiable matter, the esters present, and the degree of unsaturation of the lipid fractions. An increase in melting point was correlated with a decrease in wax unsaturation. He suggests that the decrease in unsaturation with age is caused by oxidation by atmos-
pheric oxygen. The amount of wax esters and acids, and non-wax esters increased with age. The cellular fatty acids appeared to be precursors of esters and wax fraction acids.

Kreger (67) used X-ray diffraction techniques to identify the principal components of plant surface wax. He reported the presence of long-chain members of the primary mono-alcohols, secondary alcohols, ketones, paraffins, and mono-esters.

Martin and Batt (78) dipped fresh leaves individually, with gentle agitation for 15 to 20 seconds, in four successive beakers containing ether at room temperature and then combined these solutions. The term 'waxy covering' was given to the material on or in the cutin which was extracted in this way. The term 'wax' was used for the fraction precipitated by acetone from the concentrated ether extract. The wax fraction obtained often constituted a small proportion of the total waxy covering of the leaves examined. Cotton swabs, moistened with ether, were used to wipe the surface of cauliflower and laurel leaves. Using this method, extraction from within the leaves was not likely to occur but the composition of the waxy material recovered from the leaf surface was comparable with that obtained by dipping. Cabbage and cauliflower leaves yielded less total waxy material but a higher proportion of true wax per unit area than the apple leaf. Laurel had a higher proportion of wax in the waxy covering of winter leaves than in the summer leaves.
Origin of wax

deBary (28) reported that there were different points of view concerning the origin of plant wax. One commonly held was "like other so-called secretions, that they are a product of growing cellulose membranes and cuticle." He considered that the wax substances are a reduction product of carbohydrates. In Figure 2 is a reproduction of a drawing from deBary's paper showing wax droplets in the epidermal cell wall after the surface wax had been removed and the leaf placed in hot water.

Channon and Chibnall (17) present evidence that wax is synthesized continually throughout the life of the plant in the above ground parts. They consider that the wax is produced by the protoplast and is a by-product of metabolic activity.

Kurtz (68) believed that the wax he extracted was derived from the cuticle. In his study he reports a positive correlation between the thickness of the cuticle and the wax yield. In his view only minor amounts of wax would be found in the protoplasm, although he cites other reports "that wax is steadily synthesized as the plant matures and is not catabolized."

Two recent reports lend credence to the idea that plant wax is a result of metabolic activity. Juniper (61) reports that the pre-emergence treatment of peas with trichloroacetic acid to control weeds reduced the number and form of minute wax structures on the leaf. The carbon replica technique was
used in the study as well as the determination of the contact angle of water droplets. Adamson (2) found that the waxy bloom did not develop on cauliflower and cabbage leaves when the herbicide EPTAM (ethyl N,N-di-n-propylthiocarbamate) was used as an preemergent or post-planting treatment. Both of these herbicides are known to reduce metabolic activity of plants.

Migration to surface

It is clear from deBary's (28) paper that botanists had wondered how wax got to the surface of the leaf many years before his day. There are a number of references in the literature to fine canals through the cuticle of wax-bearing plants. Dous (30) removed wax from the leaf surface and considered that he could see the openings of these canals in the surface of the cuticle. He also used a melting technique, as deBary had done, and reported that he could see wax droplets in these openings. He concluded that these canals were the pathway through which wax reached the surface.

Kreger (67) considered that wax must reach the surface in solution and that the forms assumed by the wax could, in almost all cases, be the result of crystallization of the wax. He suggests that both diffusion through the external epidermal cell wall and cuticle, as well as movement through fine canals, could occur.

Mueller et al. (87) state "--- we have found small pits or craters ---, which may be pores through which some of the
wax reaches the surface of the cuticle. These pits have not been observed on the surface of leaves which do not develop external waxy deposits." They also considered that the pits tend to show patterned distribution and that it was possible that these patterns are correlated with the areas of heavy wax accumulation. In *Musa* the columnar, or thread-like structures, seem to be associated with the large pits on the leaves, but extrusions of smaller wax masses through smooth areas was equally clear. The conclusion by these workers was that the wax was extruded in a softened form under some pressure. No evidence of the secretion of waxes in liquid form was found. Schieferstein (119) continued the study of wax deposits on leaves. He was not able to determine, using the surface replica technique, whether there were pores through the cuticle. Pits were found in both upper and lower surfaces of the cuticle but he could not determine whether they extended through the cuticle. Schieferstein and Loomis (121) repeated the study of the cuticle with cabbage. They state that there is no evidence that these are more than surface pits with a well developed cuticle in the bottom. In repeated tests with corn no pits were found at any time although all except embryonic corn leaves had surface wax deposits. They concluded that pits or canals are not required for the migration of wax to the leaf surface.

Scott et al. (123) published on their study of the epidermis of the onion in which they used both the light and
electron microscope. Ultrasonic treatment was used in some of their work to obtain fragments of cell wall and cuticle which were mounted directly on copper grids and shadowed with palladium. They concluded that the outer wall of the epidermal cells is pitted. They presume that wax precursors in liquid form extrude along the plasmodesmata through the pits in the outer wall. They were not able to detect canals in the cuticle but state; "It seems possible that transcurrent pores --- may actually be present in the cuticle but due to the inevitable contraction of the membrane when eventually observed under the electron microscope, they are not detectable. The mosaic pattern faintly indicated in certain micrographs gives support to this hypothesis. If such pores do exist, it is presumed that they would be in line with plasmodesmatal endings."

**Effect of wax layer**

Ebeling (32) reported on the role of surface tension and contact angle in the performance of insecticidal and fungicidal sprays. The technique he developed was to use a micro projector, with its optical axis horizontal, to project the outline of a water drop placed on an horizontal leaf surface. A similar study was made by Fogg (36) in which the advancing contact angle was used as a measure of the extent of wetting of the leaf surface. The magnitude of the contact angle is also of importance in determining the area of contact between a leaf surface and a drop of water. Fogg believed that the
actual area of contact must be considered in any problem concerned interchange of substances between water droplets and the cells of the leaf. According to this worker the presence of a wax coating was not essential in inhibiting wetting of the leaf surface as certain aquatic plant surfaces neither retain water nor have a superficial wax layer. His observations showed that there were diurnal variations in contact angle through a range of 30 degrees and that these variations were correlated with changes in the water content of the leaf. A wilted leaf, having a dull matte appearance, resists wetting while a turgid leaf reflects light and is more easily wet.

Staniforth and Loomis (13) have shown that the addition of substances which reduce surface tension increase the effectiveness of herbicides. Their explanation for this phenomena was that the waxy coating is not continuous and that a reduction in surface tension enables the spray particles to move down into contact with the cuticle, through which the chemical may penetrate into the leaf. Surface wax deposits can therefore be a factor in the selectivity of many herbicides.

Linskens (74) developed a quantitative method for measuring the wettability of the leaf surface by using the average value of the advancing and receding contact angles as measured with an optical device. A reading of 0° is therefore recorded for a completely wettable surface while one of 180° is obtained for a surface which cannot be wet. Those species which
had surface wax deposits had, with the exception of *Iris germanica*, average contact angles greater than 120°. Contact angles of less than 80° were obtained for those species which were known to be without surface wax.

Hall and Jones (52) report that the removal of wax by brushing, or under natural conditions by weathering, reduced the contact angle of water drops. Paired leaves of the same age, one brushed - the other not, were weighed at regular intervals. The brushed leaves lost water more rapidly than the unbrushed. They suggest "that when growth is interrupted by adverse conditions, the wax removed by weathering is not replaced quickly enough and the plant is additionally stressed through having an unusually high cuticular leaf transpiration."

Surface wax deposits are important in both the ease with which a leaf may be wet and the loss of water through the cuticle. The first is of special interest to those concerned with studies of penetration through the surface layers of leaves and of the application of protective coatings such as insecticides and fungicides.

**Cuticle**

**Physical structure**

In Brongniart's first paper (12) he presented the ideas held at that time concerning the structure of the epidermis. By the use of thin cross sections of leaves which he studied with a microscope he was able to prove that the concept of the
epidermis as a distinct layer of cells was correct. In these sections he observed the thin layer, which we now call the cuticle, over the outer wall of the epidermal cells. In additional studies he isolated this layer by immersing cabbage leaves in water for many months. The simple transparent membrane obtained was without cellular detail. In his second paper (11) the results of further experiments, in which he examined by maceration a great number of leaves of both monocotyledonous and dicotyledonous plants, were presented. This work proved the general existence of a very thin superficial layer which covered the external surface of the epidermal cells. Brongniart also reported that Professor Henslow at Cambridge had used maceration in nitric acid and separated a similar membrane from the epidermis of the corolla, the filaments of stamens, and the style of **Digitalis**. His conclusion from this and other observations was that this simple layer covers all the external surface of plants with the exception of the roots.

Lee and Priestley (73) describe the cuticle as a somewhat rigid layer which does not have cellular detail because it is formed by condensation and oxidation of the film of fatty substances which accumulate at the air-water interface of the plant surface. This concept is supported in other papers by these workers (72, 106). They also state that no cellulose is present in the cuticle. The cutinized layers as well as the layers rich in pectin are considered as part of
the outer epidermal cell wall.

Meyer (83) used a polarized light microscope in her studies of the cuticle. As a result of these she depicts the cuticle as an isotropic layer. The negative double refraction she considered to be due to the wax molecules enclosed in the cuticular layer beneath the cuticle.

Sifton (127) in his paper on the development of cuticle in leaves of Labrador tea found that water soluble stains such as ruthenium red failed to penetrate except at spots where the epidermis was broken. The cuticle was also found to be insoluble in fat solvents. This finding does not agree with that of Roberts et al. (109). These investigators used standard microchemical techniques for differentiating between cutin, cellulose, and pectic substances on sections of fresh apple leaves cut with a freezing microtome. Their photomicrographs showed that the apple leaf cuticle was not a solid mass of cutin but a laminated tissue composed of discontinuous layers of cutin, cellulose, and pectic materials.

Orgell (95) in a paper on the properties of cuticle presented a diagrammatic representation of the outer wall of an epidermal cell together with the adjacent cuticle. In it the cuticle is shown as a layer of cutin with embedded discontinuous wax lamellae. The ideas of Skoss (128) are quite similar. In his view the cuticle is composed of a framework of a polymerized fat-like substance "cutin", impregnated to a variable degrees with wax.
Roelofsen, (ill) after study with the polarizing light microscope, determined that the cuticle exhibits a weak negative double refraction. His interpretation of this was that the cuticle does not contain wax, or that the waxes are combined with the cutin which renders them insoluble.

Bolliger (?) records that he was unable to see any sort of structure in the cuticle of Philodendron scandens in sections studied with the electron microscope. In other investigations he found that following immersion of cuticle in acetone several lamellae were separated and that the number of these increased with the age of the cuticle. This phenomenon he attributes to the concentration of lipoidal droplets under the existing cuticle and the subsequent formation of a new layer of cuticle following polymerization.

Chemical composition and origin

Priestley (107), in 1921, published a paper in which he reviewed the work of a number of European workers including Chevreul, Gilson, Von Hohnel, Fremy, and Van Wisselingh. In his review he defines the properties of suberin and cutin layers as "insolubility in and impermeability to water, considerable insolubility in fatty solvents, great resistance to concentrated sulphuric acid, ready oxidation by nitric or chromic acids, and ready solubility in warm alkali; they are stained by fat stains such as Sudan III and scarlet red. -- The term suberin is due to Chevreul and was given by him to a substance insoluble in water and alcohol and constituting
seventy per cent of the substance of bottle cork (*Quercus suber*)." Gilson established the presence of several organic acids, which he termed as a class the suberogenic acids. Some of these acids, such as phellonic acid and phloionic acid he obtained pure and crystalline. Only small quantities of glycerine were found, and since the suberin was insoluble in normal fat solvents Gilson concluded that suberin could not be regarded as a typical fat. Gilson obtained his suberogenic acids from suberin by saponification and then heated them in sealed glass tubes. In this way he prepared artificially condensation products of these acids which had the properties characteristic of suberin. The conclusion was "that suberin consists largely of anhydrides of these acids, possibly together with a small proportion of the acids combined with glycerine or glycerides as true fats."

Concerning the distinction between suberin and cutin Priestley wrote that "Von Hohnel and Premy referred to suberin and cutin as definite individual substances but it is obvious in the light of Gilson's work that they are more probably aggregates or mixtures differing in their composition with variations in the original proportions of the suberogenic acids from which they are formed." In the same report Van Wisselingh is cited as writing that "The differences between the suberin and cutin of different plants, or between these two substances within the same plant will be due, in part to differences in their constituent acids, and in part to dif-
ferences in the external and internal conditions prevailing whilst these acids pass over into the form that they assume in the mature suberin or cutin lamella." At the time of this report Priestley presumed that in the future these acids would prove to be derived from some of the carbohydrates found within the plant.

Lee and Priestley (73) consider that "the appearance of the cuticle in the Angiosperm shoot indicates that, as differentiation begins, fatty substances move freely along the walls until they reach the surface, where they form a film, which undergoing condensation and oxidation gives rise to a rigid layer." Some earlier workers had claimed that the epidermal cells produced the cuticle. Lee and Priestley, however, believed that the fat derived from the epidermal cells was only part of that found in the cuticle and that additional fat reached it by migration from inner tissues along the walls. They cite the case of Jasminum in which fat deposition occurs first in the cuticle, then in the cuticularized layers of the outer epidermal wall, next in the radial walls of the epidermal cells, and finally forming a continuous layer of fat completely surrounding the cavity of the epidermal cell which had by that time lost all cell contents.

The state of combination of the fats in the walls is mainly a question of the relative solubilities of the different soaps formed by the fatty acids, according to these workers. "Potassium, magnesium, and sodium soaps are rela-
tively soluble, calcium soaps insoluble. The result that may be expected is to find the mobility of the fat, and as a consequence, the thickness of the cuticle, controlled by the relative proportion of potassium, sodium, and magnesium ions to calcium ions present in the soil solution." This theory was checked by examining the cuticle produced on fruit trees in a long term manurial experiment. Where high potassium levels prevailed the cuticle was thicker. An increase in calcium produced thinner cuticles. In studies of the cuticle of certain aquatic plants however a thicker cuticle was found on plants grown in water containing most calcium.

Lee and Priestley (73) also carried out an experiment in which cellulose plates were made by dissolving filter paper in Schweitzer's reagent and reprecipitating the cellulose with hydrochloric acid. After thoroughly washing the precipitate free of copper and acid, and compressing it into plates, a film of oil extracted from radicles of *Vicia Faba* L. was spread over three such plates. One left in sunlight in a closed Petri dish formed a film which was resistant to the action of fat solvents. In the second, which was kept in the dark, the fat layer was more readily dissolved and was less attached to the cellulose. The third plate was placed under a bell jar, in darkness, and in an atmosphere of coal gas. After several days the oil layer was little changed from its condition when placed on the cellulose. From this experiment they deduced that the coal gas had displaced the oxygen under
the bell jar and had prevented oxidation. The remainder of the experiment proved that light had accelerated the rate of oxidation.

Lee (72) by studying both the normal mature cuticle and the less developed layer found upon young shoots grown under forcing conditions, obtained data which she considered "threw light on the essential chemical nature of the cuticle and the chemical changes which are involved in its formation." In one experiment with rhubarb petioles the highest iodine value obtained, 90, was for the fat from the forced rhubarb cuticle. That from outdoor grown plants was 54. Her interpretation of this was that the fats exposed to the oxidizing and drying conditions in the open became saturated more quickly than those exposed to the damp atmosphere of the forcing sheds. In summary she wrote that "cutin is a complex mixture of fatty acids, both free and combined with alcohols, that have undergone condensation and oxidation; soaps of fatty acids and unsaponifiable material which probably contains some higher alcohols; resinous substances, and a compound giving tannin reactions. -- The preponderance of oxy-fatty acids is the result of oxidation processes taking place during the deposition of the cuticle."

The paper published by Priestley (106) in 1943 restated the contention that the cuticle is "the natural result, first, of accumulation of fatty substances at the outer air-water interface of the shoot, released mainly from epidermal proto-
plasts but also from deeper lying protoplasts; and second, of
the subsequent chemical and physical changes undergone by
such fatty substances which coalesce into an originally liq-
uid film that oxidises and 'dries' to a viscous and finally to
a firm, solid film." Priestley considered that a purely pas-
sive mechanism was sufficient to explain the accumulation of
substances which formed the cuticle. His explanation was
"metabolic activity releases fatty substances from the proto-
plasts into the walls. Thus substances such as glycerides
of fatty acids and waxes - esters of fatty acids with high
molecular alcohols - are released into walls which in this
growing region are saturated with water, and these fatty sub-
stances, made up in part of long hydrocarbon chains without
affinity for water, certainly tend to migrate to the nearest
air-water surface where they accumulate with their hydroxyl
or ester groups in the water surface and their hydrocarbon
chains in the air. The fatty acids in these substances in-
clude, as do all vegetable fats, unsaturated linkages, and
these in the air oxidize and thus gradually link up together."

Orgell (95) in 1957 agreed with Priestley that cutin was
a polymer consisting of oxidized unsaturated lipids, or as
suggested by others a poly-ester. Sifton (127), Skoss (128),
and Bolliger (7) are some of the research workers who accept
this theory of cuticle formation but not all agree that the
migration of the lipids is a passive mechanism.

The idea that some active mechanism must be present is
commonly expressed in botanical literature to explain or describe how certain structures are formed. In the case of the formation of the cuticle those who do not accept the passive mechanism described previously consider that the movement of the fatty substances occurs through special passageways and that the movement is controlled or influenced by the protoplast. Schumacher and Halbsguth (122) more than twenty-five years ago reported that they had observed plasmatic strands extending freely through the cell wall to the surface in the hyphal-like cells of *Cuscuta odorata* in the tissue of the host plant. Lambertz (69), a student of Schumacher, studied the occurrence of these plasmodesma-like structures in the outer epidermal cell walls on leaves of a number of plant species. He considered that these strands were projections of the protoplast and that they penetrated the cell wall through to directly under the cuticle. Differences in the number observed he attributed to a periodic rhythm which reached a maximum during the night. Franke (37, 38) continued this study and has made several reports on the existence of these structures which he terms ectodesmata. According to this worker ectodesmata exist in the outer walls of the epidermal cells of aerial plant organs. His most recent hypothesis is that ectodesmata are submicroscopic hollow spaces in the walls between lumen and cuticle which perhaps periodically are filled with substances excreted from the protoplasts. He does not believe that they are cytoplasmic structures.
Studies of the onion epidermis have been reported by Scott et al. (123). In this work maceration of the epidermal tissue was by means of ultrasonic treatments. The fragments of cell wall and cuticle were then mounted on copper grids, shadow cast with palladium, and studied by means of an electron microscope. Their report supports Lambertz, for they say that pits exist in the surface view of the outer wall. Plasmodesmata are also reported to occur in the outer epidermal walls of Citrus and other leaves. These workers imply that the movement of substances which form surface deposits on the outer epidermal wall takes place through these plasmodesmata.

The detailed report by Bolliger (7) was based on the results of both light and electronmicroscopical studies. He was not able to detect ectodesmata in the outer walls of any of his material nor was he able to detect any sort of structure in the cuticle by means of the electron microscope.

**Chemical stability of the cuticle**

A paper by Harris (53) records the extreme chemical stability of plant cuticle. In his studies on the classification of fossil plant species cuticle has been recovered from plants as ancient as those of the Devonian period. The cuticle he obtained is not fossil material but is actually intact normal cuticle which has remained unchanged over millions of years under anaerobic conditions. The procedure used to recover the cuticle from fossil material, such as coal, is fur-
ther evidence of chemical stability, as the material is put into a mixture of nitric acid and potassium chlorate for a few hours, rinsed and then immersed in ammonia. This process dissolves the fossil material completely and the cuticle is left intact as a transparent membrane.

Permeability of the cuticle

Botanists in the nineteenth century were primarily interested in the cuticle as a structure which restricted water loss from the mesophyll cells of the leaf. Recent investigations have been much more concerned with the effect of the cuticle on restricting the ease of penetration from the surface into the underlying cells of water, nutrients, fungicides, insecticides, and herbicides.

Priestley (106) believed that there was a gradation from the pure cellulose wall through layers containing varying quantities of pectic and fatty substances to an outermost sheet of cuticle. The cuticle was usually free from pectic compounds because of the leaching action of rain on these relatively soluble compounds. It seems that he considered the cuticle to be water permeable to a degree, but that the layers of wax deposited in the cutinized wall and the cuticle were chiefly responsible for the restriction of water movement in either direction.

A few years after this paper, Roberts et al. (109) reported that since the application to stomate-free dorsal surfaces of apple leaves of aqueous spray solutions containing
minor elements had given results, that penetration of the cuticle had been achieved. Their work revealed that there was a continuous avenue of pectinaceous substances from the exterior of the cuticle to the epidermal cells and vein extensions. They postulate that water soluble materials pass through the cuticle via this pectic pathway. Boynton (8) in his review article on nutrition by foliar application supports this contention.

Weintraub et al. (148) isolated pieces of cuticle by bacterial digestion from two species, Hedera helix and Olivio nobilis, which possess thick cuticles. These were also chosen because they bear stomates on only one surface. The cuticle was mounted in a lucite cell in such a way that one side was in contact with water and the other with air. Radioactive 2,4-D was placed on the dry side and the radioactivity of the water determined at intervals. "The maximal rates of penetration observed with the stomate bearing cuticles were of the same order as the rates of entry into intact bean leaves; this seems to support the view that the cuticle is the rate-limiting barrier in the intact leaf." Cuticle from the stomate-free surface of the leaf was only 35 per cent thicker than that from the lower surface yet there was little penetration of 2,4-D. This result they considered might be accepted as proof that the presence or absence of stomatal pores is the major factor responsible for the difference in the rate of penetration of 2,4-D through isolated cuticle. "A number of facts make it doubtful, however, that the findings
with isolated cuticles should be extrapolated to intact leaves. Preliminary results indicate that in intact leaves of Senecio, 2,4-D can be absorbed through the stomate-free surface as rapidly as through the stomate-bearing surface. There seem to be important differences also in the factors influencing penetration through isolated cuticles and entry into intact leaves. Thus, cuticular penetration is much less influenced by surfactants and not at all by pH of the applied solution."

Skoss (128) in the summary of his work reported that "From the experimental observations it appears that stomates act as the major portal of entry, regardless of the nature of the sprayed substance. Small aqueous and oil droplets were shown to be actively absorbed into a leaf when a plant is sprayed while the stomates are open and the leaves are under a slight water deficit." Skoss also stated that the penetration of a surface-applied oil into a leaf is due to the property termed "creep." That is, the oil forms a surface film "which moves through the stomatal openings by means of capillary action." This author considers that cuticular diffusion is not the main path of entry although he does agree that "if the substance is an oil, it may penetrate the cuticle by diffusion, ---. If the substance is dissolved in an aqueous carrier, it may enter through the stomates when there is a water deficit in the leaves. If the stomates are closed, the solvent will evaporate, leaving the solute incrusted on the leaf surface, to be later carried in by stomatal exudate re-
sorption, if and when it occurs."

Orgell and Weintraub (96) in their study of the influence of some ions on foliar absorption of 2,4-D mention the general agreement that effectiveness is greater at acid pH than at alkaline pH. This phenomenon has been attributed to the greater proportion of non-ionized 2,4-D molecules at lower pH values and to a more rapid penetration of the plasma membrane by an undissociated molecule than by the corresponding ion. In their experiments they found that the aqueous droplets applied to leaves usually evaporated within a half hour or less but that work reported by Weintraub et al. (148) had shown "that the bulk of foliar entry of 2,4-D occurred subsequent to that time. The residue remaining after evaporation of the droplet constitutes a medium with properties very different from those of a dilute aqueous solution. The 2,4-D and additives, together with the waxes, cutin, and other materials present at the plant surface, may be envisaged as constituting a concentrated reaction system virtually devoid of free water. Ion exchange phenomena and reactions catalyzed by surface adsorption probably are enhanced. ---. The kinetics of 2,4-D absorption will be dominated by the fact that it is present in very high concentration within a limited area, resulting in an extremely large initial concentration gradient which falls rapidly as absorption proceeds. In contrast, tissues immersed in liquid solutions are exposed to a much smaller and more constant concentration gradient."
The studies reported by Weintraub et al. (148) suggest that solution of the 2,4-D molecules in a lipoidal phase takes place especially at low pH. In their experiments on the influence of the age of leaf on rate of absorption they found that "each leaf appears to pass during its expansion, through a relatively brief stage of high absorbability following which its absorbability falls very markedly and remains relatively constant so long as the leaf retains its normal green color." The report by Schieferstein and Loomis (120) indicates that this period of "high absorbability" coincides with the period in which the cuticle has not matured and wax is still being extruded through the young, fragile cuticle. When the cuticle attains its normal thickness over the entire leaf the rate of absorption falls.

In his review on absorption and translocation of plant growth regulators van Overbeek (140) states that penetration of the leaf cuticle by chemicals is unlikely. The work by Darlington and Cirulis (26), however, was based on the acceptance of the fact of penetration through the cuticle. From the data they obtained they concluded that cuticle penetration is a diffusion process and that diffusion kinetics are applicable to the system. Yamanda et al. (153) used cuticle of tomato fruit and onion leaves isolated by enzymatic digestion in penetration studies using radioactive cations. They found that the rate of penetration through different cuticular surfaces was directly related to the extent of ion binding on the
surface opposite the site of initial entry. They postulate that the greater ion binding on the inside, compared to the outside, of cuticular membranes facilitates foliar absorption.

The paper by Bolliger (7) presents the concept that cutin is capable of swelling in water and that "a moisture content gradient exists in the cutinous layers between the dry air on the outer surface of the leaf and the watery medium in the leaf. --- Just as substances can diffuse through moistened cutin layers from the inside to the outside so must diffusion in the opposite direction be possible. However, the permeability is strongly dependent upon the lipoidal solubility of the substances passing through."

**Effect of cuticle**

The work of Brongniart (11, 12) has been mentioned earlier. In one of his experiments he proved that the essential function of the epidermis was to protect the leaf against too rapid evaporation. Lee (72) used sections of rhubarb petiole in her studies of the effect of cuticle. By comparing the water loss, after sealing the cut ends, of sections from plants grown in the forcing shed and sections from plants grown outdoors she found that the loss was less from the outdoor grown rhubarb. On the outdoor grown rhubarb the cuticle would have been more completely developed and thus to have formed a more impermeable layer.

Priestley (106) points out that from very early days attempts have been made to evaluate the comparative signifi-
cance of cuticular and stomatal transpiration. The prevalence of thick cuticles in plants of xerophytic situations seems support for the contention that the cuticle contributes to the control of water loss, however, thin cuticles occur with some frequency in such situations. He also notes that some plants common in peat bogs have thick cuticles but exhibit a comparatively high rate of water loss per unit of surface.

Another effect of the cuticle discussed by this writer is that the cuticle prevents wetting and penetrations of the cellulose walls by rain. In this way loss of soluble salts by leaching is prevented, or at least restricted.

Lee and Priestly (73) state that "the cuticle on the leaves and stems of most plants grown under normal conditions is sufficiently rigid to maintain a regular tabular form in the cells of the epidermal layer by preventing their extension, while the epidermal cells of an etiolated stem are large and irregular. --- The presence of palisade parenchyma cells stretched at right angles to the surface in strongly insolated leaves suggests that in such a leaf, further lateral stretching of the cuticle ceases to be possible at an early stage in development." Priestley (106) also considered that the presence of a well developed cuticle influenced extension in surface growth.

Watson (146) in his report on studies of the development of the epidermal cells in leaves of juvenile shoots of English ivy, Hedera helix, concluded that the cause of the waviness
of the lateral walls of the upper epidermal cells was due to the outer free wall. Through the use of differential staining techniques he demonstrated that the cuticle in the sinuses of the epidermal cells was not mature in these regions but was mature in the areas where the cuticle extended to the outer edge of the cell between the sinuses. "The difference between the hardened cuticle and the soft, fatty substances from which it develops is a question of condensation and oxidation. If we consider these changes as extending gradually over the surface of the cell, it will be evident that cell expansion will be limited at the points where the hardening first reaches the radial wall, whereas in other places the cell wall will still be expansible."

In a study of Labrador tea *Ledum groenlandicum* Sifton (127) found that an incipient cuticle covers the young leaves in buds collected in November. This cuticle was easily broken down by immersing leaves in hot dilute solutions of sodium hydroxide, which indicated that polymerization had not progressed very far. There was a gradual increase in resistance to disintegration of the cuticle with older leaves which showed that the degree of bonding increased gradually. This Sifton considered sufficient to account for the fact that structures surrounded by the young cuticle continue to grow, although the fully formed cuticle would be expected to render such growth difficult.

The work of Martens (77) on the influence of the cuticle
on the staminate hairs of *Tradescantia* may be extrapolated to a certain extent. In studies using plasmolysis of the staminate hairs he was able to observe separation of the cuticle from the cellulose wall. The extreme plasticity of the walls when freed of the layer of cuticle is added proof that the mature cuticle influences the growth of the adjacent wall.

Skoss (128) believed the role of the cuticle in spray penetration to be that of an impermeable barrier to some inorganic and organic salts. The investigations by Orgell (95), Orgell and Weintraub (96), and Darlington and Cirulis (26) indicate that the cuticle can be considered as a limiting system or mechanism which controls the entry of ions and molecules into the leaf.

Another effect of leaf cuticle was studied by Roberts and Martin (110) in their investigations into withertip disease in limes. Their work indicates that cuticle development, as assessed by cutin deposition, is an important factor in protecting lime leaves from invasion by the disease organism *Gloeosporium limetticola*. They state, "the lime leaf cuticle, when sufficiently developed, provides an effective mechanical barrier to invasion."

The Cell Wall

Brongniart's reports (11, 12) may again be used as the starting point of the literature review on this portion of the subject. In his second paper (11) he states that the outer
wall is generally much thicker than the other walls of the epidermal cells of the leaf. deBary (28) published a series of camera lucida drawings of epidermal cells showing the thicker outer wall of the epidermal cells of a number of plant species. Since deBary's day both the structure and the way in which this outer wall grows has been the subject of much research and speculation.

**Structure of the cell wall**

The structure of the cell wall has been studied by means of histological staining techniques for many years. In the paper by Priestley (106) he states that "the outer epidermal wall is relatively complex in nature and shows gradation, usually from a pure cellulose wall, facing the epidermal protoplast through layers containing varying quantities of pectic substances and fatty substances to an outermost sheet of cuticle free from cellulose." This description is one which has been accepted by most botanists.

In 1864 Nageli (92) proposed that the cellulose portion of the wall consists of elongated, crystalline, submicroscopic units which he called micelles. Subsequently, other investigators have used techniques such as X-ray diffraction analysis, chemical analysis, and infra-red spectrophotometry to study the structural components of the wall. The micelles suggested by Nageli have been considered to be cellulose groups made up of parallel chains of glucose residues. A great deal of work has been done to determine how many glucose
molecules comprise each micelle.

Sponsler (129, 130), following his studies on the molecular structure of the cell wall and the mechanism of cell wall formation, considered that cellulose is not present as micelles of discrete length but that the units are attached to each other and form "chains of indefinite length." These, in turn, have many side chains and form a three-dimensional space lattice. Many different terms have been used for this lattice, for example, Siegel (126) in his monograph refers to them as "micellar frameworks." Others use the term fibrils for this same lattice, while a few reserve the term fibrils for larger units enclosed by films of various kinds of cementing materials.

More recent work, using shadowed cell wall preparations and the electron microscope, by Muhlethaler (88, 89, 90), Preston (103), Myers et al. (91), Probine and Preston (108), Roelofsen (111), Roelofsen and Houwink (112), Green and Chapman (47), Steward and Muhlethaler (134) and others show fibrils or microfibrils in all cell wall material. This evidence from electron microscopical studies supports the X-ray diffraction studies discussed by Siegel (126). Siegel also corroborates the earlier work of Sponsler that "cellulose has a degree of polymerization of 1400-10,000, hence molecules ranging in length from 7000-50,000 Å (0.7-5 μ)." This writer also reports that "By taking the utmost precautions for the exclusion of oxygen during cuprammonium extraction, a mol-
ecular weight of over 20,000,000 has been obtained, corre-
sponding to --- a molecular length of at least 0.05 μμ."

The pectinaceous component of the cell wall mentioned in
the description by Priestley (106) has been studied using
histological staining techniques such as those of Roberts,
Southwick and Palmiter (109), and Sifton (127). Ruthenium
red is the favored stain but some doubt exists concerning the
precision with which pectic compounds can be detected with
this stain. Considered as a qualitative method, however,
there is general agreement with Johansen (59) that it has
merit. Chemical procedures for the study of pectins have
also been used extensively. In the monograph on plant cell
walls by Siegel (126) an excellent summary of this work is
presented. He states that "major differences in all principal
wall components exist. The most significant distinction lies
in the pectic substances which have been specifically impli-
cated in the hormonal regulation of cell extension. In the
older analyses, the pectic substances formed an appreciable
part of the wall, whereas the more recent analyses demonstrate
only trace amounts. It has been claimed that ammonium oxalate
solubility, which is commonly adduced as evidence for the
presence of pectins, may be misleading as polysaccharides
other than pectin may also appear in this extractant in ap-
preciable quantities." Later he says that "The pectins are
characterized by their content of ester methyl groups. The
ideal limits for these polymers are unesterified polyglacto-
uronic acid and the fully esterified molecule which has no acidic properties. The intermediates with varying proportions of free and esterified carboxyl groups represent the pectic and pectinic acids." Siegel also records that "From X-ray diffraction studies, it has been established that the pectic acids differ in spatial arrangement from other polysaccharides such as cellulose."

The hemicellulose fraction isolated is not homogeneous either physically or chemically. Some are thought to be important as structural elements of the polymers.

Lignin, another component of the cell wall "may constitute half, or even more, of the cell wall substance in older tissues, or may be indetectable in youthful tissues. --- X-ray diffraction and polarized light techniques have been used in studies which demonstrate that isolated lignins lack the highly ordered crystalline structure found in many polysaccharides." Other data suggest "a three-dimensional polymer, perhaps a reticulum."

Growth of the cell wall

The theories concerning cell wall growth which appear to have the most support at this time can be called (1) tip growth, (2) mosaic growth, (3) multi-net growth, and (4) marginal growth. A brief review of these theories has been included in the paper by Roelofsen and Houwink (112). They report that tip growth has been demonstrated in the parenchyma cells and epidermal cells of Avena coleoptiles, in cambium
cells, and in the filamentous algae *Spirogyra*. Their description of this method of growth is "the tips of the cells are opened and the cytoplasm apparently oozes out of the cell. It weaves its wall. First, more or less longitudinal fibrils are deposited. These act as a kind of warp and are interwoven with a weft of mainly transverse fibrils, which soon outnumber the longitudinal ones, thus producing the negative birefringence of the fully grown primary cell wall." This type of growth cannot take place in a sporangiophore when it carries a sporangium nor in the sporangium-free growth stage, for the whole growth-zone, including the extreme tip, is negatively birefringent. The theory of tip growth does not apply in growing *Tradescantia* staminal hairs as the hair is a chain of cells not enclosed in a sheath as is *Spirogyra*. The cells, therefore, cannot have growing tips. These authors discount the claim that tip growth takes place in the hairs of cotton, as they have observed that not only are all parts of the cell wall negatively birefringent but "the axial and the transverse fibrils are not interwoven, but occur in different layers."

In other cells growth is not an addition of new cell wall areas, but is a true cell wall extension, presumably due to a pushing apart of fibrils by local plasmatic growth. In these spots the cellulose structure is temporarily completely perforated. New fibrils grow or are inserted in these areas. This type of intussusception growth was named mosaic-growth
by Frey-Wyssling (41, 44) and his collaborators. The conspicuous perforations seen in electron micrographs of cell wall preparations have been interpreted as proof for this theory. Muhlethaler (89, 90) and Wardrop (143) have also published micrographs showing these pits. For one paper Muhlethaler (89) arranged a series of micrographs showing the fibrillar nature of the cellulose about and within such perforations or pits. This series, he considers, shows the developmental stages of pits and the subsequent filling in of the pit as new cellulose microfibrils are deposited.

All of these investigators assume that the protoplast controls this stretching of the wall and the deposition of fibrils. This "vitalistic" view is not shared by all botanists. Colvin (19) has shown micrographs which appear to be similar to those of Muhlethaler, Roelofsen, and Wardrop but these had been made during studies of artificially produced polymerized material. According to his views the mechanism responsible for the "arrangement" of the fibrils is to be found in the realm of physical chemistry and is a purely mechanistic phenomenon.

Roelofsen and Houwink (112) propose a third theory for cell wall growth which they term "multi-net growth." In this type of growth the cell wall is characterized by the occurrence of a great number of submicroscopic tears or meshes distributed uniformly in the cell wall in all the growth regions of the cell. These tears or meshes occur in separate
layers and hence the cell wall is not perforated. In contrast
to the theory of mosaic growth the meshes do not fill in with
fibrils but persist and extend. They may fill in with non-
fibrillar material. According to the authors "the majority
of new fibrils will be deposited on the inside, adjacent to
the outer protoplasm layer." Their concept of the way ex-
tension of the cell wall takes place is as follows. "Owing
to cell wall tension and, maybe, assisted by local plasm
growth into the cell wall, the small bundles of newly deposi-
ted interwined fibrils occurring on the inside of the cell wall
will be split locally, thus producing the transversely orient-
ted superficial meshes. Since these meshes are not filled in
with new fibrils, axial cell wall extension elongates them
more and more in axial direction. Meanwhile new fibrils are
deposited on the inside and the extending mesh is therefore
gradually shifted to the outside of the cell wall, probably
being more and more filled in with incrusting materials."
Later they state "the 'nets' are not separated, but exten-
sively interwoven. There are no distinct layers, but a grad-
ual change in structure. If our conception is right, there
is apposition-growth if one considers the fibrillar material
only, but it is intussusception-growth if one considers the
incrusting material which fills in the meshes."

In his work with *Avena* coleoptiles Wardrop (143), in part,
supports the idea that cell enlargement is controlled by the
synthesis of cell wall material at synthetic centers (pit
fields and plasmodesmata) distributed over the cell surface. While he avoids a definite statement, it can be presumed that at the time of writing this paper the author agreed with the theory of mosaic growth. After further studies of parenchyma isolated from *Avena* coleoptile segments Wardrop (144) discarded both the tip growth and mosaic growth theories and accepted the multi-net theory of growth. In his studies using labelled glucose and autoradiographs he found that "there was no concentration of radioactive material at the cell tips and labelled cellulose appears to be uniformly distributed in the cell wall. Electron micrographs of similar material show that the cellulose microfibrils are almost transversely oriented on the inner surface of the cell wall but are considerably dispersed from this direction on the outer surface."

There have been a number of studies made of cell wall growth using autoradiographic methods. Those of Bayley et al. (6) and Setterfield and Bayley (125) are typical of most of these studies. These workers placed segments cut from rapidly growing etiolated oat coleoptiles on the surface of dilute manganese sulphate for three hours. The segments were then placed in a buffered growth medium containing indolacetic acid and 2 per cent tritiated sucrose. After the selected time interval the segments were killed in 70 per cent ethanol. Autoradiographs were prepared using both sections from parafin embedded material and squashed segments. After ten days exposure the stripped film was developed, mounted in water, and
observed under both bright field and phase contrast illumination. The uniform distribution of silver grains was considered as proof that the labelled sucrose, and hence the newly deposited cell wall material, had been added to the cell wall over the entire surface. An objection to this interpretation is that many of the tritium atoms recorded on the autoradiographs may have been exchanged from the labelled sucrose to carbon atoms present in the wall.

Probine and Preston (108) continued their work with *Nitella opaca*. The orientation of microfibrils in the inner lamellae as contrasted with that of the microfibrils in the outer layers in this organism led them to state that "Multinet growth may well therefore be involved in the extension of this particular cell wall." In the process of multinet growth "the whole wall is considered to stretch passively and therefore becomes thinner." This reduction in thickness is counterbalanced by simultaneous apposition of new wall lamella on the inner face of the wall, which then comes in turn under stress.

The fourth concept concerning cell wall growth may be called marginal growth and has been presented by Schieferstein and Loomis (120). In their work on the development of the cuticular layers of the leaf epidermis the micrographs obtained were considered evidence for this theory. They claim "When young, but nonexpanding, leaves were dewaxed by wiping with cotton wool the wax was not replaced. When the wax was removed from growing regions, it was not replaced in the center.
of the cells, but bands of normal wax deposits were formed at their margins. It is concluded that surface wax in maize is extruded only through the very young cell wall and cuticle, and that new wall, cuticle, and surface wax deposits are formed at the margins of the outer walls of growing epidermal cells. This growth pattern would account for the unimpeded growth of epidermal cells partially covered with a non-plastic cuticular layer."

Source of cell wall constituents

There has been general acceptance amongst botanists for almost a century that the constituents of the cell walls are provided in some way by the activity of the protoplast. A few, such as deBary (28), have considered that some material is synthesized within the wall itself. These two ideas are not incompatible and recent work has indicated that both may be correct.

The organization of these constituents has been attributed, over the same period of time, to the phenomenon of protoplasmic streaming. This concept is still considered valid by many botanists although others reject any such "vitalistic" interpretation. Proponents of the protoplasmic streaming concept point to the apparent concentration of cytoplasm in the region of secondary wall growth which has been observed with the light microscope. Recent work making use of the greater magnification possible with the electron microscope has not lent support to this idea. Wooding and Northcote (152) and
Cronshaw and Bouck (24) state that there was no differential accumulation of cytoplasmic components in the region of secondary wall growth in the material they studied with the electron microscope. Esau et al. (34) confirmed this observation in their studies on the cytology of differentiating tracheary elements.

After the discovery of microtubules in plant cells by Ledbetter and Porter (71) there was some speculation concerning their function. Cronshaw and Bouck (24) in their studies of differentiating xylem elements found that the microtubules occurred in groups and that these groups were oriented parallel to the secondary thickenings. These authors state "Our results support the view that the morphological association of the 'microtubules' with the developing cell wall thickening may have a functional significance, especially with respect to the orientation of the microfibrils." Esau et al. (34, 35) agree that the microtubules are directly concerned with the construction of the secondary wall thickening but state that the endoplasmic reticulum is also involved. In their work with tracheary elements they "found endoplasmic membranes differentially applied to those parts of sieve plates that later would have been perforated. --- however, the presence of the endoplasmic membranes appeared to be concerned with a prevention of wall deposition rather than with contribution to wall growth." Cronshaw and Bouck (24), however, state that "at no stage does there seem to be a preferred orienta-
tion of the endoplasmic reticulum, either with respect to the primary wall structure or the secondary wall thickenings." They believe that the endoplasmic reticulum may have a function in some way connected with the synthetic mechanism of cell wall deposition.

The work reported by Frey-Wyssling et al. (42) sheds some light on the synthetic function of the endoplasmic reticulum. In their paper they say "The so-called spherosomes in plant cells evolve from vesicles produced by the endoplasmic reticulum. In tissues which store fat, they differentiate into oil droplets. --- They stain with all known fat dyes and therefore must be considered lipidic bodies, but in addition they react with cationic fluorochromes (janus green malachite green, etc.) indicating a possible presence of anionic proteins." Wooding and Northcote (152) agree with the concept that the spherosomes are the plant organelles mainly concerned with lipid synthesis.

There is a considerable body of evidence that the Golgi vesicles are the primary source of the secondary wall constituents. Mollenhauer et al. (86) were the first to report that the contents of the dictyosome vesicles may be incorporated into the cell wall. In root cap cells they found that the contents of the vesicles accumulated between the plasmalemma and the wall and became confluent with the wall. Wooding and Northcote (152) in studies of the developing xylem of *Acer pseudoplatanus* have shown that some material is added
to the wall in discrete packets or vesicles. Frey-Wyssling et al. (43) studied the formation of the cell plate and reached the conclusion that the Golgi apparatus is an organelle capable of synthesizing substances which serve as precursors for cell wall matrix. Cronshaw and Bouck (24), Esau et al. (34), Buvat (15), and Horner et al. (56) have added additional evidence on the role of the Golgi vesicles. Horner et al., after study of spore development in the liverwort Riccardia pinguicula, report that "wall formation is under the exclusive control of the spore protoplast, "-- that the --" cell plates form from coalescing Golgi vesicles," and that the pattern on the outer surface of the spores is determined by the wall against which it is formed."

These authors also believe that the "contents of the vesicles change successively during wall formation, corresponding to the different wall layers being formed." The presence of Golgi vesicles with both electron transparent (Esau et al., 34) and electron opaque contents (Cronshaw and Bouck, 24) is evidence that the vesicles contain and presumably produce more than one kind of wall constituent. Mollenhauer and Whaley (85) suggest that in the root cap cells and epidermis of Zea mays root tip the material contributed by the vesicles becomes part of the mucilage present in the walls of these tissues. Whaley and Mollenhauer (149) and Frey-Wyssling et al. (43) believe that the initial contribution of the dictyosomes would be largely that of pectic mate-
rials. Frey-Wyssling et al. (43) and Cronshaw and Bouck (24) state that later contributions of the vesicles are concerned with incorporation of material into the matrix of the wall.

The influence of the plasmalemma on movement of the vesicles to the cell wall is not quite clear. The plasmalemma is not a static structure, according to Esau et al. (35) but permits passage of materials out of the protoplast. Buvat (15), Cronshaw and Bouck (24), Esau et al. (34, 35), Horner et al. (56), Mollenhauer et al. (86), and Pickett-Heaps and Northcote (101) all report evidence that the spherical Golgi vesicles move through the plasmalemma and that their content accumulates between the membrane and the cell wall.

The fate of the membrane about each vesicle is uncertain. Whaley and Mollenhauer (149), Frey-Wyssling et al. (43) and Esau et al. (34) believe that fusion takes place and that the vesicle membranes become part of the plasmalemma. Buvat (15) reports that the plasmalemma is a unit membrane, i.e. double membrane, while the membrane of the vesicle is single. The two membranes could not, therefore, be identical and fuse readily. He believes that the plasmalemma ruptures and permits passage of the vesicle into the region or space between the plasmalemma and the cell wall.

Most of the workers mentioned in this section imply that the substances contained within the Golgi vesicles are precursors which become part of the cell wall matrix after passage through the plasmalemma. The work of Colvin and Dennis
(20) on the wall of *Acetobacter xylinum* has shown that cells are capable of producing an enzyme system and precursors which can move outside the cell and synthesize cellulose microfibrils.

The addition of material in discrete particles against the inner surface of the wall is considered evidence by Esau et al. (34), Pickett-Heaps and Northcote (101), and others that growth is by apposition. If one accepts the idea that the wall is a rigid structure which prevents or restricts the passage of small aggregations or molecules through the wall it becomes difficult to understand how growth in area takes place in outer epidermal cell walls without any apparent reduction in thickness. In their study of pollen grain walls Rowley, Muhlethaler, and Frey-Wyssling (113) found submicroscopic channels in several species of grass. Martens (77) proposed, some years ago, that the plant cell wall is a much more elastic structure than some suppose, and that it is not as restrictive as these workers imply.
MATERIALS AND METHODS

Replica Studies

Plant material

The plants used for this portion of the project were from both greenhouse and field grown specimens. When leaves were taken for study, three types were selected: (1) young recently expanded leaves, (2) leaves which were completely expanded but did not show signs of weathering, and (3) mature leaves. To avoid possible artifacts caused by application of fungicides and insecticides, field grown samples were taken only from areas which had not been sprayed. Fumigation was used to control insects in the greenhouse in which most of the other plants were grown. When other material was taken, only leaves which had emerged after the application of sprays were used.

Each sample was carefully removed so that the surface was not touched. Each blade was then pinned to a solid sheet of cork fastened inside a plastic tray. All leaves were used within a short time of collection.

Double replicas

In order to study the outer surface of the leaf epidermis a double replica technique similar to that used by Mueller et al. (87), and Schieferstein and Loomis (121) was used. By means of this technique a cast or form is made, this cast is then used to form a thin Formvar replica of the leaf surface,
and then a heavy metal is used to shadow the irregularities on the surface of the replica. Differences in areas of metal accumulation and the thickness of the deposition affects the electron beam and makes possible observations on the fluorescent screen in the electron microscope, and also the production of a photographic image on either film or a plate.

A considerable amount of difficulty was encountered in trying to follow the procedure described by Schieferstein (121). The most serious of these were, first, keeping the polyvinyl alcohol solution confined to one area of the leaf, and secondly, the 0.05 per cent solution of Formvar in dioxane did not produce replicas which were stable in the electron beam. These problems were solved by adoption of the following procedure:

(1) The leaf was pinned to a piece of cork thick enough and large enough to hold it flat.

(2) A one per cent solution of the wetting agent, Tergitol 7, was used to wet the leaf surface. This was then rinsed off by dipping in distilled water.

(3) A plastic washer with a 1.5 cm. opening was then fastened to the surface with four pins. This device made it possible to keep the leaf blade flat, kept the polyvinyl alcohol in place, and made it easier to remove the cast from the leaf.
(4) A small quantity of either a 15 or 20 per cent aqueous solution of polyvinyl alcohol (DuPont Elvanol 51-05) was poured on to the moist leaf surface.

(5) The cast was usually dry by the next day and was removed from the leaf by lifting the plastic washer.

(6) The cast was then removed from the washer and pinned to a cork float. These were then placed, cast side down, in small jars containing a 1 to 2 mixture of chloroform and xylene. Two washes, each of 30 minutes, were sufficient to remove the surface waxes retained in the cast.

(7) After the cast had dried a positive replica was formed by applying a small quantity of 0.25 or 0.5 per cent solution of Formvar dissolved in ethylene dichloride. The amount used was only enough to wet the surface. The Formvar film formed on drying was approximately the same thickness as that used when coating copper grids. The 0.25 per cent Formvar solution produced a thinner film.

(8) A thin protective layer of celloidin dissolved in amyl acetate was applied to the Formvar to make a three layered cast.
(9) To mount the replicas on copper grids the layered casts were cut into squares slightly larger than the diameter of the grid. The polyvinyl alcohol was dissolved from these squares by placing them in small dishes of distilled water.

(10) Copper grids were cleaned by washing in two changes of carbon tetrachloride and dried on filter paper in a petri dish.

(11) Uncoated copper grids were placed on copper mesh in a petri dish. After removal of the polyvinyl alcohol the small squares were placed concave side down on the grids and allowed to dry until the next day. The sections were usually flat on the grid by morning.

(12) The celloidin layer was then removed by dissolving it with amyl acetate placed on the sections with a pipette. Amyl acetate was added as required until only the Formvar layer was left.

(13) When dry the specimen grids were placed in a semi-circle on a glass microscope slide which in turn was placed in a vacuum evaporator so that all grids were the same distance from the point of the filament. The height of the filament, and the distance of the grids from the filament were set to give shadowing at from $15^\circ$ to $45^\circ$. 
A short length (eight turns about the filament) of platinum or platinum/palladium wire was wrapped about the tungsten wire filament at the point of the filament. After the bell jar was evacuated to operating vacuum the current was turned on and the metal evaporated to completion.

The specimens were then examined in an electron microscope and representative areas were recorded on photographic plates.

**Carbon replicas**

Bradley (9), and Bradley and Juniper (10) have developed a carbon replica technique which they claim reproduces the fine structure of the leaf surface with greater freedom from artifacts than any other method. Their method, and several minor variations, were used in this study to make comparisons between it and the double replica technique.

The procedure developed by these workers was as follows:

1. The leaf is cut into pieces of convenient size and mounted on a glass slide with cellulose adhesive tape.

2. The slide is placed in a vacuum evaporator and when the vacuum is satisfactory about 15 m of carbon is deposited. This is estimated by using a spot of oil on a white porcelain slide as a check. When the carbon coating on the porcelain slide has a slight brownish tinge the carbon layer
is thick enough.

(3) The leaf is then removed and a layer of Formvar (a 2 per cent solution in chloroform) is applied over the carbon.

(4) When dry, a layer of Bedacryl 122x (a 5 to 7 per cent solution in benzene) is applied over the Formvar.

(5) A strip of cellulose tape is pressed on to the Bedacryl surface when it has dried.

(6) The leaf is then removed.

(7) The "sandwich" is then placed tape side down in a dish and acetone is used to dissolve the Bedacryl layer.

(8) Copper specimen support grids are slipped between the tape and the Formvar carbon film.

(9) The strip of tape with the grids in position is removed from the acetone and allowed to dry.

(10) The grids can then be lifted from the tape without damaging the overlying film.

(11) A chloroform bath is used to remove the Formvar, leaving the carbon replica on the grid.

(12) The replica is finally shadowed at a fairly high angle. Forty-five degrees is suitable.

The modifications made in using this technique were (1) to shadow cast the leaf surface with palladium or platinum/palladium before deposition of the carbon layer, and (2) to
cut the three layered sandwich into small squares, which
separated from the leaf surface quite readily and were then
placed directly on the grids. The Bedacryl layer and then
the Formvar layer were removed by applying the solvents with
a pipette. As soon as the grid had dried it was placed in a
small dust-proof box until examination in the electron micro­
scope.

Studies of wax

Petroleum ether was used to remove wax from leaves of
cabbage seedlings in a manner similar to that described by
Martin and Batt (78). This, briefly, was to dip fresh leaves
with gentle agitation for 15 to 20 seconds in four successive
beakers containing ether at room temperature. The solution
from the four beakers was mixed together and the ether al­
lowed to evaporate. A paper towel was fixed in place above
the beaker to exclude dust. The contents of the beaker were
swirled at intervals to remove the wax from the walls of the
beaker. The solvent was allowed to evaporate completely.

Some of the dry wax was placed on a glass slide, a cover
slip was placed over it, and ether was run in from the side
of the cover slip. The crystal aggregates, which formed as
the solvent evaporated, were studied with a light microscope.

Portions of the dry wax material were dissolved in ether
and acetone was added. The precipitate produced is considered
"wax" by Martin and Batt (78) and Channon and Chibnall (17)
while the acetone soluble fraction is the non-wax fraction of
the "waxy covering." A glass pipette was used to place drops of solvent containing wax on the surface of copper grids which had been covered with a Formvar film and then coated with carbon. These grids were then shadow-cast in the same way as the leaf surface replicas.

Studies of the Cuticle

Differential staining

The use of differential staining techniques by many investigators has established that the outermost continuous layer of the epidermis is the cuticle. The reports of Priestley (106, 107), Roberts et al. (109) and Sifton (127) are typical of this area of study. The fatty substances which make up the cuticle have an affinity for the specific dyes Sudan III and IV. The work of Roberts et al. (109) and Johansen (59) has shown that a layer of pectic substances is present between the cuticle and the outer wall of the epidermal cell. The presence of this layer is shown by its affinity for the dye ruthenium red. In this study these simple tests were repeated with sections cut from fresh onion leaves. In addition, sections were cut from specimens of onion leaves which had been fixed in osmium tetroxide and embedded in Epon according to the procedure used by Luft (75). The sections were mounted on glass slides and then placed in a Coplin jar filled with a one per cent aqueous solution of Nile blue. This dye has an affinity for lipids.
and hence the cuticle appeared dark blue when examined with a light microscope.

**Separation of cuticle from epidermal cells**

The cuticle has been separated from the epidermal cells by soaking leaves in water for several weeks, by heating in dilute sodium hydroxide followed by immersion in hydrochloric acid, by the use of ultrasonic vibrators, and by the use of pectic enzymes. A method similar to that used by Baker et al. (4) and Orgell (94) was considered to produce the least damage to the cuticle.

A citrate buffer solution was made up and adjusted to pH 5 with dilute hydrochloric acid. Two enzyme solutions were prepared by using 10 and 15 grams of Pectinol 10M (Rohm & Haas) each in 100 ml of the citrate buffer. A merthiolate solution (0.1 gram in 50 ml distilled water) was prepared for use to prevent bacterial contamination. Five drops of this mixture were added to each container of 20 ml of the enzyme mixture. Pieces of leaves from cabbage seedlings, onions, candle plant (*Kleinia articulata*), *Fatsheadera Lizei*, and English box were placed in the enzyme mixture and kept at room temperature (22°C.). After 18 hours the cuticle had separated sufficiently from most of the leaves to make removal possible with a toothpick and pair of tweezers. The pieces of cuticle were left in the mixture for an additional 24 hours to ensure that the pectic material holding isolated cells or cell fragments was dissolved. The pieces of cuticle were then washed
in distilled water and transferred to containers of distilled water and merthiolate.

Pieces of cuticle were washed again in distilled water, examined under a binocular to check freedom from cellular debris, and then were cut into small squares slightly larger than the copper grids. These pieces were "floated" on the surface of distilled water and were picked up on individual grids. After drying on filter paper, those grids on which the cuticle appeared flat were shadow-cast with platinum/palladium in the same way as the surface replicas of the previous study. An adhesive made by dissolving the adhesive on a short length of cellophane tape with chloroform, as described by Pease (100), was tried on some of the grids.

Electronmicrographs were prepared of typical areas on the specimens. Intermediate negatives were made of selected plates before printing.

Studies of Thin Sections

The basic principle of the electron microscope is to focus a stream of electrons on the specimen in a way analogous to that in which the light microscope focuses light rays. Differences in electron density across the specimen impede or scatter the electrons so that an image is formed on the fluorescent viewing screen. When a suitable subject is found the image can be recorded on a photographic film or plate.

The clarity, or resolution, of micrographs obtained with
modern electron microscopes is limited by both the performance of the microscope and by the nature of the specimen. Adjustment of the instrument is covered adequately in manuals provided by the manufacturer, or in a more general way in books such as those of Kay (65) and Pease (100). Details of the procedures adopted in this study will be found in Appendix A Epon embedding and Appendix B Selectron: Methacrylate embedding. These procedures vary to some extent from those used for studies of animal tissue but the differences are not great.

**Thin sectioning**

In making replicas the thickness of the specimen to be studied is determined by the concentration of the Formvar used in replicating the surface or in the amount of carbon deposited during some procedures. The heavy metal used to shadow the replica is a minor factor. To study the ultrastructure of most biological material, however, it is necessary to kill or fix the specimen, embed it in a suitable medium, and then cut thin sections with a microtome. The sections can then be mounted on a support of some kind and studied under a microscope. Books such as those of Sass (117) and Johansen (59) make the proven techniques of histologists and light microscopists available to all. Several books on the techniques used in electron microscopy are now available, good examples are those of Kay (65), Pease (100), and Mercer and Birbeck (82).

The technique of cutting thin sections is considered first simply because of its importance. The thickness of the sec-
tions is critical for two reasons; First, the electrons emitted by the gun have low penetrating power and the number which finally penetrates thicker sections does not produce a satisfactory image on the screen because of inelastic scatter. Secondly, the resolving power of the electron microscope is such that it would not be possible to interpret the layers of structural material revealed. These matters are discussed in the papers by Cosslett (22, 23) and by Peachy (99). An accepted 'rule of thumb' is that section thickness should not be greater than ten times the desired micrograph resolution for optimum results. Electron microscopes are now available which are capable of a resolving power of better than 10A. A specimen thickness of 100A is therefore required to make use of the full potential of the instrument. It has been claimed that sections of 60A have been cut but it is now believed that the original measurements of section thickness were in error and that the sections previously presumed to be 100 to 200A thick were in reality closer to 600 to 900A (Peachy, 99). Most of the acceptable micrographs obtained in the present study were from sections which were in the light gold range or approximately 900A thick.

Fixation

The fixatives generally used in optical microscopy are not satisfactory for specimens to be studied in the electron microscope. The reason for this is that finer structures are observed in the electron microscope and these must be
preserved if acceptable micrographs are to be produced.

Many fixatives have been tried but buffered osmium tetroxide, as used in the technique developed by Palade, has been the most useful. This technique, and several variants of it are discussed in the texts by Kay (65) and Pease (100). In addition to its activity as a fixative osmium tetroxide stains selectively the phospholipid membranes of the cells. This fixative does not penetrate tissue very rapidly and for this reason the pieces must be either small cubes or thin slices, and fixation should be carried out at room temperature.

Mollenhauer (84) has described the use of potassium permanganate which is excellent for the preservation of cell membranes. Holt and Hicks (55) found that the morphological and biochemical integrity of the fixed tissue was best preserved by using four per cent formaldehyde, buffered at pH 7.2 with phosphate buffer, and containing 7.4 per cent sucrose.

More recently the studies of Sabatini et al. (115, 116), Ledbetter and Gunning (70), and Ledbetter and Porter (71) have established that glutaraldehyde is an effective fixative. This compound is easy and safe to handle, penetrates tissue with sufficient rapidity to make practical the use of larger specimen blocks or pieces, and the preservation of fine structure is superior to that of the previously used fixatives. It is now standard practice to post fix with osmium tetroxide after glutaraldehyde fixation.
Dehydration

This procedure is similar to that used in the dehydration of tissue which is to be studied with the optical microscope. All the free water is removed from the specimen and is replaced by alcohol or acetone. It has been found that shorter times of immersion in each of the graded series of alcohol or acetone are necessary to avoid excessive extraction of cell components. Polyester resins such as Vestopal are not soluble in alcohol and hence acetone is used when the specimen is to be embedded in this agent.

Infiltration and embedding

Most of the plastics or resins used for embedding are highly viscous. To ensure penetration of the tissue, so that the plastic fills all the space within the individual cells, suitable infiltration procedures must be used. Details of satisfactory procedures will be found in the reference books mentioned previously. The ideal is to have polymerization take place throughout the specimen at the same time and to effect polymerization without a change of volume.

Various mixtures of methyl, ethyl, and butyl methacrylate were tried but without success. Araldite, as used by Glauert and Glauert (45), and Vestopal (Ryter and Kellenberger, 114) were more successful. The technique developed by Luft (75) concerning the use of Epon is now standard in many laboratories. A procedure using Selectron: Methacrylate mixtures was also tried and found satisfactory. These last two techniques were
both satisfactory and were used in this study.

In a study of this type the "positioning" of the specimen before sectioning was a problem. Two methods were used which were satisfactory. The first was to embed in a capsule or aluminum boat, cut out and trim a block of plastic containing the specimen, and clamp it in a vise-like specimen holder or chuck. The second method was to prepare some large blocks of the desired resin and drill a small hole in the end after removal of the gelatin capsule. Instead of using small cubes of tissue, thin slices of leaf specimens were cut. Before the final stage of embedding the drilled blocks were placed in capsules in an upright position. A fine pipette was used to fill each hole with the resin mixture and then the thin specimen slices were picked up on a toothpick and placed in the block. A gelatin cap was placed on each capsule before the preparations were placed in the oven.

Sectioning

Each specimen block was examined under a binocular and two of each series were selected. The blocks were trimmed and sections cut on a Huxley, LKB, or Reichert microtome.

Mounting and staining of sections

Formvar coated grids and uncoated 300 to 400 mesh copper grids were used. The problem of getting sufficient contrast was a serious problem through most of the study. Reducing the aperture size as suggested by Valentine (138, 139) did not help. The staining procedures suggested by Parsons (97),
Peachy (98), and Watson (145) were not satisfactory. Most of the useful sections were stained with lead according to the method described by Karnovsky (64) or by Venable and Coggeshall (141). Most specimen material was also immersed in uranyl acetate for one to two hours, between fixation and dehydration. This stain is considered as an unspecific stain but is useful as it increases the general contrast of sections. The technique of Stempak and Ward (133) of staining sectioned material mounted on grids with uranyl acetate in methanol was not used.

Examination with the electron microscope

An RCA EMU 2A microscope was used at the start of the study. A Philips 75 was the only instrument available for most of the time but for the latter part of the study a Philips 200 and an RCA EMU 3F were used.

Several thousand sections were examined to study the structure of the cuticle and the outer epidermal wall. All micrograph plates were examined under maximum enlargement and selected areas were studied further under a binocular dissecting microscope. Prints were made, following the usual techniques for photographic enlargement and printing.
RESULTS

Replica Studies

Replicas of leaf surfaces prepared by the polyvinyl alcohol cast method described and used by Mueller et al. (87) and Schieferstein (118, 119), and by the carbon replica technique used by Juniper (62) gave dissimilar images but each technique by itself gave reproducible results. The micrographs produced in this portion of the study were similar to those published by these investigators. In Figures 1 to 4 examples from Schieferstein's and Juniper's papers may be compared with corresponding micrographs prepared during the present study.

The amount and distribution of surface wax varied widely over the species studied. However, where several species of the same genus were studied the wax pattern and appearance of individual wax aggregates was similar within the genus (Figures 5, 6) provided the method of preparation was the same.

An experiment was carried out using cabbage seedling leaves to determine the influence of vacuum evaporation as well as the use of a dilute wetting agent upon the delicate wax layer. Portions of the same leaves, or portions of paired leaves from the same seedling, were prepared for the different treatments. On some, polyvinyl alcohol casts were made, both with and without the use of a dilute wetting agent, in the usual way under normal room conditions. Other leaf samples were
fastened to glass microscope slides and placed in a vacuum evaporator. After exposure to the same degree of vacuum, for the same length of time as required for carbon evaporation, the samples were removed and polyvinyl alcohol casts made on the surfaces, again with and without the dilute wetting agent. Four portions of each of these replicas were mounted on copper grids and shadowed as described in the preceding section. Wax appeared as crystals (Figures 7, 8) when the wetting agent was not used, whether or not the intact leaf had been subjected to high vacuum. When a wetting agent was used, the usual amorphous aggregates were observed in both specimens that had been subjected to vacuum and those that had not (Figures 9, 10).

A few replicas were made, using both techniques, on leaves which were taken from plants grown in the open. The replicas shown in Figures 11 and 12 are from leaves of oak leaf goose-foot (Chenopodium glaucum) found growing on the research station at Bradford, Ontario.

Leaves of seedling cabbage plants which were free of all spray residue were harvested from greenhouse grown plants. The wax was removed by the method described previously (Martin and Batt, 78) and studied in several ways.

When the petroleum ether was allowed to evaporate at room temperature, the solids, which would be the "waxy covering" according to Martin and Batt, (78) were deposited on the inner surface of the beaker. When studied under a binocular dissecting microscope much of the deposit was seen to be definitely
shaped crystals in a great range of sizes.

A small portion of this dry wax was placed on a glass microscope slide and covered with a cover slip. The wax was then dissolved by allowing petroleum ether to run under the cover slip. As the solvent evaporated crystals formed near the edge. These were variable in both shape and size but were readily identified as crystals under the light microscope. This confirmed the finding described by Johansen (59).

Drops of solution containing wax dissolved in petroleum ether were placed on the surface of Formvar coated grids which had been coated with a carbon film. These were allowed to dry at room temperature. After shadowing with platinum they were examined in the electron microscope. The results were not conclusive.

Several genera and species similar to those described by deBary were included in this study. No evidence of "rods" as described by him were seen. Figures 13 to 16 show reproductions from plates in the original paper of some of his work. Figures 17 and 18 present some of the results of the present study.

All micrographs of replicas made by both techniques fail to show openings in the cuticle which bear any relationship to the wax pattern. This was confirmed with the original plates for Figures 19 to 24, as well as several others, when they were examined under a binocular dissecting microscope.

Schieferstein (118) has shown micrographs with an epi-
dermal cell border zone in which there is evidence of a greater redisposition of wax after a period of growth following removal of the existing surface wax by wiping. Two micrographs are presented from this study (Figures 25, 26) which show differences in wax deposition at the margin of two epidermal cells without any previous removal of wax. The significance of this will be discussed in the section on cuticle.

Studies of the Cuticle

Some freehand sections were cut from fresh onion and cabbage leaves. The cuticle turned black when stained with Sudan III. This is considered a specific stain for the identification of fatty substances (Conn, 21).

Differential staining on similar fresh leaf sections confirmed that a pectic layer was present between the cuticle and the outer wall of the epidermal cells. A concentration of the red stain was apparent in this region.

To determine whether the cuticle remained intact after the specimen had been through the fixation, dehydration, and embedding procedures required before study in the electron microscope a number of thick sections were cut from a specimen of onion leaf tissue embedded in Epon. Nile blue was used as described by McGee Russell in the discussion which follows the paper by Mercer (81). The cuticle sections were similar in outline to those seen in the fresh specimens and the cuticle appeared to be a uniform dark blue from the inner to the outer surface.
The cuticle was examined on all the sections studied in the electron microscope. The material selected for study was from nine species including one used by Franke (37) in his work. No plasmodesmata, nor indication of them could be seen in any of the hundreds of sections studied with the electron microscope. Micrographs which show the fine structure of the cuticle in section are presented in Figures 27 to 29.

The successful removal of intact cuticle, by the use of pectic enzymes, from cabbage and onion leaves made it possible to study both the inner and outer surfaces of comparatively large areas of cuticle from these plants. Figures 30 to 33 present representative micrographs which show that they are free of openings or pores.

No pores were detected in any of the replicas through areas of the cuticle which could be seen between the wax protuberances. In the micrographs of young seedling cabbage leaves a number of pits can be seen. The majority of these seem to be shallow indentations and do not appear to penetrate the cuticle (Figure 19).

Studies of Thin Sections

Thin sections of leaf epidermal cells of seven species of dicotyledonous and two monocotyledonous species were studied. Onion and cabbage were used most extensively but others included were corn, cauliflower, pepper, passion flower (Passiflora coerulea), oak leaf goosefoot (Chenopodium glaucum),
plantain (Plantago major), and a tobacco species (Nicotiana glauca).

The outer wall of these epidermal cells was much thicker than the other walls. A relatively low magnification is used in the micrograph shown in Figure 34, of onion epidermal cells, which shows that the preservation of wall and cuticle was very good. In Figure 35 the definite layering of the wall can be seen. The arrangement of the first formed microfibrils is shown in Figure 36 while the progressive change to a more orderly orientation is shown in Figure 37.

In Figures 38 to 45 are samples of micrographs which show typical outer wall structure in the other eight species studied. There is no evidence of canals nor ectodesmata in any of the material. Dark lines seen in some of the sections studied earlier with the Philips 75 were proven to be folds in the section when examined with the Philips 200, which has much better resolving power (Figure 34).

Studies of Sections of Young Tissue

The micrographs shown in Figures 46 to 49 show some of the cytoplasmic organelles which are present in the young epidermal cells. Dictyosomes and vesicles are clearly shown to be present. Note the vesicles near the ends of the Golgi apparatus in Figure 46. In Figure 47 a vesicle can be seen between the intact plasmalemma and the wall. The significance of this will be discussed in the next section. Figures 48 and
and 49 show micrographs with similar cytoplasmic organization in other species.
DISCUSSION

Replica Studies

There is a difference of opinion between those who have used the carbon replica technique and those who have used the polyvinyl alcohol cast replica technique for studies of the leaf surface. The first group claim that the replica produced by evaporating a thin layer of carbon over the surface is a true replica, while the polyvinyl alcohol cast replica is an artifact due to the solvent action of the water and aqueous solution of the casting material, or to the detergent used to wet the surface. The second group contend that the use of a vacuum evaporator in the carbon replica technique is more likely to create an artifact as the leaf is desiccated in the high vacuum produced in the device used for deposition of the carbon layer. The removal of water from the leaf sample, including water entrapped in the surface wax, would create changes in the shapes and surfaces of the wax aggregates, and the high temperature required to evaporate carbon, would create forces which would distort and melt the fine structure of the wax aggregates.

In this study it was clearly demonstrated that each technique, by itself, gives reproducible results. It was also shown that the wax pattern and appearance of wax aggregates is similar when several species within the same genus are compared using the same preparatory methods. This suggests a
similarity in the composition of the wax mixtures in that genus. There is, therefore, the probability that the composition of leaf surface waxes follows phylogenetic patterns. Baker and Martin (5) have made a similar suggestion with respect to the chemical composition of cutins.

An experiment was carried out to determine the influence of vacuum evaporation and the use of a dilute wetting agent. Since wax appeared as crystals when the wetting agent was not used, whether or not the leaf surface had been subjected to high vacuum, the only conclusion possible is that the wetting agent alters the shape of the wax aggregates. The presence of crystals seen on the micrographs of replicas made with and without exposure to high vacuum can also be interpreted as proof that the high vacuum itself was not a factor in producing the crystalline shapes. The micrographs of replicas made with polyvinyl alcohol, but without the wetting agent, are not as clear as those made with the wetting agent. This is due to the fact that polyvinyl alcohol, used without the wetting agent, does not fill the spaces between the wax aggregates down to the cuticle, trapping air between the leaf surface and the polyvinyl alcohol.

Most of the work on surface replicas has been done on greenhouse grown plants. There is some possibility that wax may not crystallize on the surface of leaves growing outdoors. One suggestion is that the leaves, or leaf parts, on plants growing in a greenhouse have been heated by the sun in such a
way that crystallization of wax has taken place. Carbon replicas made on the leaf from a plant grown in the open have a definite crystalline pattern. Apparently the crystalline pattern is not greatly affected by the environment in the greenhouse.

It should be informative to learn the shape taken by wax, removed from the leaf surface with a solvent, when the solvent evaporates. Does it form crystals or does it form an amorphous mass? When the petroleum ether used as the solvent was allowed to evaporate slowly crystals were formed on the inner surface of the beaker. Crystals were also formed as the solvent was allowed to evaporate slowly from under the edge of the cover slip. These were mostly needle-like when examined with the light microscope.

When drops of solution, containing cabbage leaf surface wax dissolved in petroleum ether, were placed on the surface of the carbon coated grid evaporation of the solvent was rapid. After shadowing and examination in the electron microscope the image formed did not resemble either the polyvinyl alcohol replicas or the carbon replicas of cabbage leaf surfaces. Neither did it resemble the crystals formed when the solvent was evaporated from wax in the beaker or under the cover slip. It seems that the rate of evaporation is important, and in this experiment the rate was too rapid to allow crystal formation.

Most workers in the past have felt that the shape of the
rod-like wax elements shown by deBary (28) was due to extrusion of wax through pores or openings through the cuticle. Hall and Donaldson (50, 51) report that the pores do exist in the clover and cabbage leaves they have studied using a carbon replica technique on leaves pre-shadowed with uranium oxide. Weber (147) on the other hand, failed to find pores in the cuticle of castor bean, white pine, and cactus (Opuntia), and of wax palm (Copernicia cerifera) and concluded that the wax is secreted in a liquid form which infiltrates through the epidermal wall and cuticle. Comparison of the micrographs made by both the polyvinyl alcohol cast and carbon replica techniques during the present study with the figures from deBary's paper fails to show the rod-like elements he depicts.

Examination of all the micrographs of replicas made by both techniques failed to reveal anything which could be considered to be pores which bear any relationship to the wax pattern. The upper and lower surfaces of isolated cabbage cuticle were also shown to be free of pores although a number of shallow pits were seen.

All the cells of the leaves of a lupine (Lupinus albus) and sunflower (Helianthus annuus) are formed before the leaf unfolds (Sutherland, 136). As the leaf grows there is a rapid increase in both size and surface area of the epidermal cells. Schieferstein's (118) micrographs show redisposition of wax near the margins of the cell in leaves which are still expanding. In Figure 25 the micrograph shows a different pattern
along the cell margin of a young oak leaf goosefoot leaf. Figure 26 shows the margin between two cells of Chamaedorea. The plant from which the leaf specimen was taken had been kept in a cool storage greenhouse and transferred a short time before to a warmer display greenhouse to stimulate growth. The new wax was found only near the edge of the cells. Schieferstein attributed his results to the presence of unhardened areas at the margins of the cuticle covering each epidermal cell. This concept is accepted by the author.

The micrographs shown on many of the plates reveal that the wax layer is discontinuous and is partly responsible for a rough surface on the leaf. Adam (1) claims that, in addition to the hydrophobic nature of wax, roughened surfaces increase the frequency of regularly spaced air gaps, increasing the contact angle of water drops, and thus increasing water repellancy. The waxy covering is therefore an important factor in determining whether spray droplets will wet a leaf or roll off. The effect of surfactants, or wetting agents, on foliar absorption has been studied by Staniforth and Loomis (131) and by Freed and Montgomery (39). These authors found that the reduction in surface tension was an important factor in absorption. This is in agreement with the theories of Adam (1).

Studies of the Cuticle

The paper by Lee and Priestley (73) and the later one by Priestley (106) were discussed in the literature review. Their
conclusion that the cuticle is formed through the condensation and oxidation of a film of fatty substances which accumulate on the leaf surface is generally accepted. Baker et al. (3) have studied the chemical composition of cuticle on the fruit of apple and their finding supports Priestley's ideas on cuticle formation. The reaction of Sudan III on freehand sections of fresh onion and cabbage leaves supported these ideas in that the cuticle, of the two species used extensively in this study had a positive reaction in this standard test for lipoidal substances. In similar sections the pectic layer between the cuticle and the cell wall was revealed by intense staining of this zone by ruthenium red. Pectic material is generally present in plant cell walls (Kertesz, 66) in this region and this made it possible to use pectic enzymes to remove the cuticle from leaves with a minimum of damage to the thin cuticle. The micrographs of the heavy metal-shadowed cuticle in Figures 32 and 33 show that in cabbage there are no passageways through the cuticle. The undersurface of the onion cuticle (Figure 31) has structures that appear to be openings, but when thin cross sections of onion cuticle are examined (Figures 27 to 29) it is seen that these "openings" do not penetrate the mature cuticle.

The staining procedures using Nile blue and Sudan III on thick sections of onion epidermal wall embedded in Epon are interpreted as proof that the preparatory procedures have not
destroyed parts of the cuticle. The sections studied under the electron microscope were therefore considered representative of the structure of the fresh cuticle.

The sections of cuticle shown in Figures 27 to 29 are typical of the cuticle of onion. There is no evidence of passageways of any kind to the outer surface corresponding to the ectodesmata described by Franke (37, 38). These results are typical of the many other micrographs taken of sectioned epidermal cells of all species studied, as well as visual observations on many more sections. Franke used plantain (Plantago major) as an example of a plant which has prominent ectodesmata and this species was included in the present study.

The ideas of Lee and Priestley (73) concerning the origin of the cuticular components are still valid. They believed that the fats derived from the epidermal cells were only a part of that found in the cuticle and that additional fats reached the air-water interface of the plant surface from underlying cells by a process of simple diffusion or migration along the walls. These fatty precursors are believed to be acted upon by localized specific enzymes and converted to cutin (Heulin and Gallop, 54).

The effect of the cuticle on limiting the rate of absorption of external substances into the leaf has been studied by a number of workers (Norman et al. 93, and Woodford et al., 151). Cuticular penetration occurred in the studies of Dybing and Currier (31) but the rate was relatively slow. Stomatal
penetration by aqueous solutions occurred rapidly if an ef-
ficient surfactant was used at the proper concentration. Many
leaves, however, do not have stomates on the upper surface and
yet penetration does take place. The cuticle shown in Figures
28 and 29 illustrate that there are localized regions where
masses of fibrillar wall material penetrate into the cuticle.
These diminish in diameter the farther they penetrate and stop
before they have gone through one third the thickness. One
can see numerous extremely fine elements with the staining
properties of the wall microfibrils apparently throughout the
cuticle. While these are not in any sense the "ectodesms"
described by Franke they may provide a certain permeability to
the cuticle and may lose this property with increased age.
Support for this concept is also provided by the work of
Schieferstein (118) on the presence of wax near the margins
of expanding leaves and by the present evidence (Figures 25
and 26) of a similar pattern on leaves from plants in which
there had been a change in the rate of growth.

Studies of the Outer Epidermal Wall

Thin sections of the epidermal cells of the nine species
studied revealed that, in addition to the lack of ectodesmata
in the cuticle ectodesmata were not present in any of the
outer epidermal walls. Some plasmodesmata were seen in the
other walls similar to those described by Strugger (135) but
these were much smaller in section than the ectodesmata des-
cribed by Franke (37). In the cell wall of radish root hairs, which are modified epidermal cells, Dawes and Bowler (27) claim that there are pores in the cellulose wall which increase in number toward the base of the hair.

The micrographs of onion epidermis (Figures 34 to 37) show that there is a definite layering of the wall. Near the cuticle (Figures 36, 37) the microfibrils are not in an orderly array. As one examines the wall structure toward the protoplast, it can be seen that there is a gradual change to an orderly arrangement of microfibrils within each layer. This is in agreement with the findings of a number of workers who have studied wall growth in widely divergent species (Green, 46; Preston, 104; Houwink and Roelofsen, 57; Frei et al., 40; and Stecher, 132). This change, which goes on in the relatively thick outer wall, has been the subject of a great deal of work and conjecture. Thimann and Bonner (137) over thirty years ago suggested that auxin, in some indirect way, influenced the extensibility of the wall. About ten years ago Cleland and Bonner (18) suggested that the initial step in cellular expansion may be an auxin induced loosening of the cell wall which allows the cell to take up water and expand osmotically.

Until recently the way in which the precursors for wall synthesis reached the wall could only be studied indirectly. Setterfield and Bayley (124) used tritium and autoradiography to study oat coleoptile cell walls. They deduced that growth
was by apposition. Esau (33) has presented a summary of the many ideas held over a period of years. Mercer (80) deals briefly with the growth of the cell wall. Some ideas are that the orientation of the microfibrils is caused by strain on the entire wall. The difficulty with this is that it does not explain a regular change in orientation such as that which we see in the micrographs (Figures 34 to 37). Preston (104) believes that in seeking the mechanism of microfibril orientation it is necessary to go back to the cytoplasmic surface. He also considers that stretching of the wall provides room within the wall for the deposition of micelles.

Studies of Sections of Young Tissue

The micrographs shown in Figures 46 to 49 show some of the cytoplasmic organelles generally considered responsible for the synthesis of much of the material added to the wall and cuticle (Mollenhauer et al., 86; Wooding and Northcote, 152; Esau et al., 34, 35; Cronshaw and Bouck, 24; and Horner et al., 56). This study revealed an abundance of Golgi apparatus and associated vesicles adjacent to the wall and this lends support to the hypothesis that vesicles are produced by the Golgi apparatus and move through the plasmalemma to a position against the cell wall. Presumably further synthesis then takes place resulting finally in the incorporation of new material into the wall and cuticle probably by apposition.
SUMMARY

1. The adaxial surfaces of the leaves of Allium cepa, Brassica oleracea var capitata, Brassica oleracea var botrytis, Chenopodium glaucum, Chamaedorea sp., Musa sp., Strelitzia reginae, and Dianthus caryophyllus were studied with the electron microscope. The carbon replica and the polyvinyl alcohol cast replica techniques were used in the study of both the surface of the cuticle and the distribution and form of the surface wax.

2. A comparison of the carbon replica technique with the polyvinyl alcohol cast technique has been made. Definite crystals were observed on the micrographs of carbon replicas but the edges of the wax aggregates seen on the polyvinyl alcohol replicas were rounded. Polyvinyl alcohol casts, with and without the use of a wetting agent, were made on normal cabbage leaves as well as on leaves which had been subjected to a high vacuum. The only differences observed that were considered significant suggest that leaf surface wax elements are present in crystalline form on the surface of cabbage seedlings and that these crystals may be modified by the wetting agent.

3. Wax was obtained from the surfaces of cabbage leaves by dipping the leaves in petroleum ether. When the solvent was allowed to evaporate slowly, the solid wax left was crystalline.
4. Occasional pit-like 'pores' were seen on the surface replicas of the cuticle but these bore no relationship to the wax pattern typical of the species. None of the 'pores' or ectodesmata passed through the cuticle as described by some workers.

5. Samples of intact thin cuticle were obtained by immersion of leaf samples from young cabbage and onion leaves, in a solution of a commercial preparation of pectinase. This treatment dissolved the pectic layer between the cuticle and the outer surface of the epidermal cell wall. Portions of these samples were placed on grids and shadowed with platinum/palladium prior to examination in the electron microscope. No 'pores' were found in the cabbage cuticle. The inner surface of onion cuticle appeared to have openings but these did not show in the upper surface.

6. Leaf samples from Zea mays, Passiflora coerulea, Nicotiana glauca, Capsicum frutescens, Brassica oleracea var capitata, Brassica oleracea var botrytis, Chenopodium glaucum, Plantago major, and Allium cepa were fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated, and embedded in Epon or a Selectron/Methacrylate mixture. Uranyl acetate was used before the start of the dehydration series in some preparations. All sections were stained with lead. Sections of cuticle embedded in Epon were stained with Nile blue. Examination with the light microscope revealed that the cuticle had remained intact through the fixation, dehydration, and
embedding procedures. The cuticle on fresh freehand sections of cabbage and onion leaves gave a positive reaction when stained with Sudan III, a specific stain for fatty substances.

7. Neither the cuticle nor the cell wall of the nine species sectioned had ectodesmata. An anastomosing system of fine tubules or lipid-permeable fibrillar elements 50 to 60 Å in diameter, were seen in the onion cuticle. It is considered a reasonable hypothesis that the wax components diffuse through this system to the leaf surface and form crystals as the solvent evaporates. In mature cuticle this system is blocked and surface wax deposition is restricted.

8. Micrographs of sections of young epidermal cells show Golgi bodies, vesicles, and other cytoplasmic organelles. These results, together with a more orderly orientation of microfibrils in the inner portion of the cell wall than in the outer, can be interpreted as support for the hypothesis that growth of the cell wall is by apposition.
LITERATURE CITED


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APPENDIX A: FIXATION, DEHYDRATION, AND EMBEDDING SCHEDULE FOR EPON

Schedule I. Osmium fixation

A. One to two hours at room temperature in one percent osmium tetroxide in distilled water in veronal acetate buffer as follows:

1. Veronal acetate buffer
   - sodium barbital: 2.88 grams
   - sodium acetate: 1.15 grams
   - distilled water to make: 100 ml

2. Final solution
   - 2 percent osmium tetroxide: 25 ml
   - buffer as in (1): 10 ml
   - 0.1 N HCl: 10 ml
   - check pH and if necessary adjust to 7.2 to 7.4 and make up to 50 ml with distilled water

3. Specimen preparation
   Use small blocks of tissue or thin slices not more than .5 mm thick - or - use small discs cut from the leaf with a specially sharpened hypodermic needle

4. Aspirate and swirl the liquid in the vial to sink specimen and get rid of air bubbles in the tissue

B. Dehydration and embedding

1. Rinse three times in buffer at same temperature as fixative
2. 10 minutes each in 30 and 50 percent alcohol
3. 15 minutes each in 70, 95, 100 percent alcohol
4. 15 minutes in 100 percent alcohol
5. Pour off and immerse the tissue in two changes of propylene oxide (under fume hood)
6. Pour off and add 2 ml fresh propylene oxide
7. Add equal quantity of Epon resin (See C.) containing accelerator
8. Swirl or put on mechanical rotator to mix. One hour
9. Add another equivalent volume of the resin mixture. Mix on rotator at slow speed. Two to four hours
10. Place fresh resin mixture in capsules or aluminum boats
11. Transfer specimens with wooden toothpick to surface of new resin mixture and allow to sink
12. Polymerize at 35°C for 12 hours, 45°C for 12 hours, and 60°C for three days

C. Epon mixture (Luft, 75)

1. Mixture A
   62 ml Epon 812
   100 ml DDSA

2. Mixture B
   100 ml Epon 812
   89 ml NMA
Schedule II. Glutaraldehyde fixation with post-fixation in osmium tetroxide (Sabatini et al., 115)

A. Two hours in 5 percent glutaraldehyde buffered at pH 7.3 at room temperature

1. Buffered glutaraldehyde in phosphate buffer

- 0.25 M Na$_2$HPO$_4$·12H$_2$O 35 ml
- 0.25 M KH$_2$PO$_4$ 15 ml
- distilled water 50 ml
- 25 percent glutaraldehyde 25 ml

2. Phosphate buffer

- (a) 7.1 grams Na$_2$HPO$_4$ in 500 ml distilled water (0.1 M)
- (b) 7.8 grams KH$_2$PO$_4$ in 500 ml distilled water (0.1 M)

Keep separate as stock
To use take 7 parts (a) and 3 parts (b) to get 0.1 M solution at pH 7.2 to 7.4

3. Sucrose washing buffer

- 6.84 grams sucrose in 100 ml of phosphate buffer (Equivalent to molarity of 0.2 M sucrose)

B. Wash in two changes sucrose washing buffer

C. Post fix in osmium (Schedule II) for 2 hours

D. Wash in buffer solution

E. Proceed with dehydration and embed as in Schedule I
APPENDIX B: FIXATION, DEHYDRATION, AND EMBEDDING SCHEDULE FOR SELECTRON/METHACRYLATE

A. Preparation of monomer mixture

Selectron/Methacrylate 50:50

1. Remove hydroquinone stabilizer from a mixture of 9 parts butyl methacrylate and 1 part methyl methacrylate by shaking, in a separatory funnel, with 30% NaOH (equal volumes) - remove amber layer - repeat until NaOH layer is water clear

2. Wash the mixture 3 times with distilled water

3. Filter the washed methacrylate mixture through CaSO₄ supported by two layers of Whatman #1 filter paper

4. Collect in amber bottle with screw cap

5. Add benzoyl peroxide to a concentration of 4 percent

6. Store in refrigerator

7. Allow methacrylate and selectron to reach room temperature before opening

8. In a bottle, containing a 1/4" layer of molecular sieve (washed with acetone and dried in oven). Mix 50 ml methacrylate and 50 ml "Selectron" RS5003. Store in refrigerator

9. Warm to room temperature before use. This constitutes the "monomer mixture" with a final concentration of 2 percent benzoyl peroxide
B. Fixation - in glutaraldehyde followed by post-fixation in osmium tetroxide as in Appendix A

C. Uranyl acetate stain. Use saturated solution for 2 hours

D. Dehydration at room temperature
   30 minutes in 70 percent acetone; 30 minutes in 90 percent acetone; three changes 30 minutes each in 100 percent acetone

E. Infiltration and embedding
   1 hour in 50:50 acetone: Selectron/Methacrylate mixture
   24 hours at room temperature with fresh mixture as above with continuous mixing on rotor
   Transfer with toothpick to fresh mixture in capsules
   Polymerize at 45°C for 12 hours; at 60°C for two or three days
Key to All Labels

C - Cuticle
CR - Crystal
CW - Cell wall
CY - Cytoplasm
G - Golgi apparatus
M - Microfibrils
P - 'Pits'
SP - Stainable projection
V - Vesicles
Figure 1. Polyvinyl alcohol replica of surface of cabbage leaf shadowed with platinum/palladium. Micrograph from intermediate negative. White areas represent wax. 15,000X.

Figure 2. Polyvinyl alcohol replica of surface of cabbage leaf from Schieferstein and Loomis, 121 showing similarity to Figure 1. Approximately 7,500X.

Figure 3. Carbon replica of surface of cabbage seedling leaf. Micrograph from intermediate negative. Note crystalline wax. 15,500X.

Figure 4. Carbon replica of surface of cabbage leaf. From Juniper's paper. Note similarity to Figure 3. 15,500X.
Figure 5. Polyvinyl alcohol replica of young cauliflower seedling leaf. 20,000X.

Figure 6. Polyvinyl alcohol replica of young cabbage seedling leaf. 20,000X.
Figure 7. Polyvinyl alcohol replica of cabbage leaf made without a wetting agent before application of casting media. Leaf not exposed to high vacuum before casting. Note crystals (CR). 32,500X.

Figure 8. Polyvinyl alcohol replica of portion of same cabbage leaf as in Figure 7. No wetting agent used but leaf subject to high vacuum before casting. Note crystals (CR). 32,500X.
Figure 9. Polyvinyl alcohol replica of similar cabbage leaf surface as in Figures 7, 8, and 10. Wetting agent used before casting. Leaf not exposed to high vacuum. 32,500X.

Figure 10. Polyvinyl alcohol replica of similar cabbage leaf surface made with the use of a wetting agent after exposure to high vacuum. 32,500X.
Figure 11. Carbon replica of oak leaf goosefoot leaf surface from plant growing outside. Note crystalline appearance of wax. 25,000X.

Figure 12. Polyvinyl alcohol replica of oak leaf goosefoot leaf surface from plant growing outside. 25,000X.
Figure 13. Reproduction of drawing of *Strelitzia ovata* leaf surface showing guard cell and wax on adjacent area. From deBary, 28.

Figure 14. Reproduction of drawing of cross section through portion of a leaf of *Strelitzia ovata* showing wax rodlets on surface. From deBary, 28.

Figure 15. Reproduction of portion of leaf surface from *Heliconia farinosa* showing guard cells and wax rodlets on adjacent area. From deBary, 28.

Figure 16. Wax rodlets of *Heliconia farinosa*. From deBary, 28.
Figure 17. Polyvinyl alcohol replica of surface of leaf of *Strelitzia reginae* showing folds and sparse wax distribution. 32,500X.

Figure 18. Polyvinyl alcohol replica of surface of *Chamaedorea* leaf, showing area near stomate. 32,500X.
Figure 19. Polyvinyl alcohol replica of young cabbage seedling leaf surface. Note 'pits' (P.). 32,500X.

Figure 20. Polyvinyl alcohol replica of young cauliflower seedling leaf. 12,000X.

Figure 21. Polyvinyl alcohol replica of young onion leaf. 12,000X.
Figure 22. Polyvinyl alcohol replica of mature carnation leaf from greenhouse. 32,500X.

Figure 23. Polyvinyl alcohol replica of banana leaf surface from greenhouse grown plant. 20,000X.

Figure 24. Polyvinyl alcohol replica of oak leaf goosefoot leaf surface from greenhouse. 25,000X.
Figure 25. Polyvinyl alcohol replica of oak leaf goosefoot showing distribution of wax near cell margins. 32,500X.

Figure 26. Polyvinyl alcohol replica of Chamaedorea leaf showing wax near cell margins. 32,500X.
Figure 27. Section through outer wall of onion epidermal cell showing relationship of cuticle and wall. Cell wall (CW), Cuticle (C). Fixed in glutaraldehyde. Post-fixed in osmium tetroxide. Embedded in Selectron/Methacrylate. Stained with uranyl acetate after fixation. Section stained with lead. 12,500X.

Figure 28. Section through outer wall of onion epidermal cell. Preparation the same as for Figure 27. Note texture of cuticle. 45,000X.

Figure 29. Enlargement from same specimen as above. Note stainable projection (SP) of cell wall and fine structure of cuticle. 177,500X.
Figure 30. Outer surface of cuticle from onion leaf. Cuticle shadowed with platinum/palladium. Cuticle removed by pectinase digestion. 32,500X.

Figure 31. Inside surface of cuticle from onion leaf. Cuticle from onion leaf. Cuticle prepared as for Figure 30. Note arrow to 'hole' which corresponds to the part filled by the stainable projection shown in Figure 29. 32,500X.
Figure 32. Inner surface of cuticle from cabbage seedling leaf. Cuticle prepared as for Figure 30. 32,500X.

Figure 33. Outer surface of cuticle from cabbage seedling leaf with all wax removed. 32,500X.
Figure 34. Low power micrograph of cross section of onion epidermal cells showing cuticle (C), cell wall (CW), inner walls of epidermal cells, and thickness of outer wall. Note arrow on fold. Osmium tetroxide fixation and embedded in Selectron/Methacrylate. Stained with aqueous solution of uranyl acetate before dehydration. Section stained with lead. 5,250X.

Figure 35. Micrograph of same section as in Figure 34. Note cuticle (C) and layering of cell wall (CW). 20,000X.
Figure 36. Cross section through outer wall of onion epidermal cell. Glutaraldehyde fixation with post fixation in osmium tetroxide. Stained with aqueous uranyl acetate before dehydration. Embedded in Selectron/Methacrylate. Section stained with lead. Note fine structure of cuticle (C) and unoriented distribution of microfibrils near cuticle. 76,250X.

Figure 37. Micrograph from same specimen as Figure 36. Note early orientation of microfibrils (M).
Figure 38. Section through outer epidermal wall of cabbage leaf. Glutaraldehyde fixation with post-fixation in osmium tetroxide. Embedded in Epon. Stained with lead. Note structure of cell wall (CW) and organelles in cytoplasm. 50,000X.

Figure 39. Section through outer epidermal wall of oak leaf goosefoot leaf. Preparation similar to that for Figure 38. 50,000X.

Figure 40. Section through outer epidermal wall of plantain (Plantago major). Note cell wall (CW). 50,000X.

Figure 41. Section through outer epidermal wall of young cauliflower. Fixation in osmium tetroxide and embedded in Epon. Stained with lead. 50,000X.
Figure 42. *Corn seedling* (*Zea mays*). Section of outer epidermal wall. Preparation the same as for Figure 38. Note wall (CW) and vesicle (V). 50,000X.

Figure 43. *Passiflora coerulea*. Section of outer epidermal wall. Preparation the same as for Figure 38. 50,000X.

Figure 44. *Pepper* (*Capsicum frutescens*). Section of outer epidermal wall. Fixed in osmium and embedded in Epon. Stained in aqueous uranyl acetate before dehydration. Section stained in lead. 50,000X.

Figure 45. *Nicotiana glauca*. Section of outer epidermal wall. Preparation of specimen the same as for Figure 38. Note cytoplasm (CY) and organelles. 50,000X.
Figure 46. Corn seedling. All specimens on this plate are from the same sample which was fixed in glutaraldehyde, post fixed in osmium tetroxide, and stained after sectioning with lead. Note Golgi apparatus (G) and vesicles (V). 102,500X.

Figure 47. Corn seedling. Note vesicle (V) between wall and plasmalemma. 102,500X.

Figure 48. Corn seedling. Note vesicles (V) on Golgi apparatus. 102,500X.

Figure 49. Corn seedling. Note cell wall (CW) and cytoplasmic structures. 102,500X.