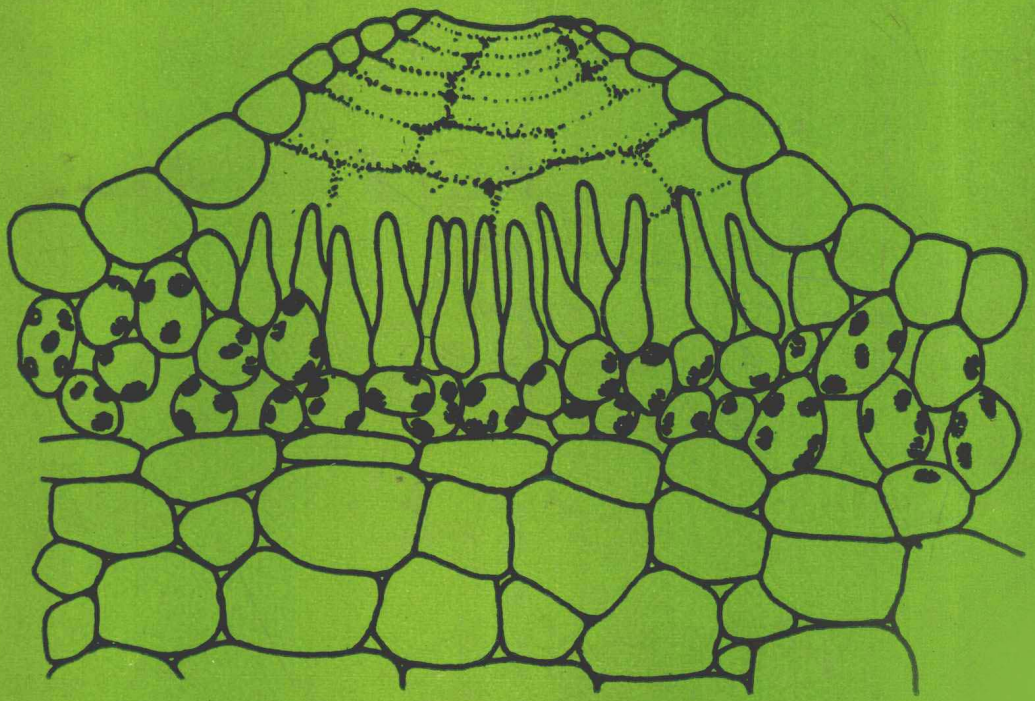
PLANT SURFACES



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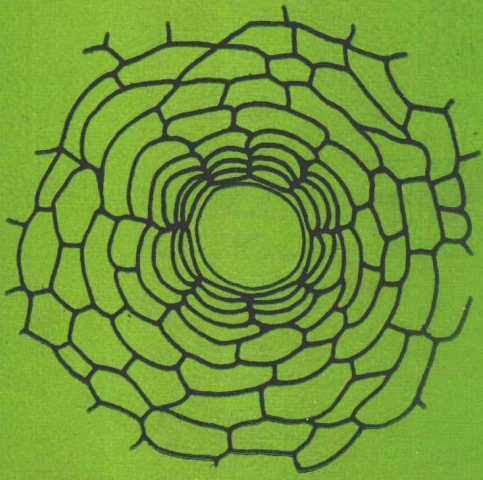

Plant Surfaces

B. E. Juniper
Botany School, University of Oxford

C. E. Jeffree
Botany Department, University of Edinburgh

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Preface

The plant cuticle, that delicate skin over the cellular tissue, was first observed 200 years ago. It was, as might be expected, the first plant surface examined in detail. But with a few exceptions, notably the pioneering biochemistry of A. C. Chibnall in the 1930s, the chemistry of this skin was too complex and its detail too fine for much advance to be made before gas–liquid chromatography and electron microscopy.

We now know something about the biosynthesis of the various polymers which make up the cuticle. Model systems can simulate its development. We know something too about the ways in which the environment can modify both this and other plant surfaces and the extent to which such surfaces are under genetic control. We know very little, as yet, of the ways the various chemical components of the plant's surface move from their synthesis within the living cells out to their point of deposition in or on the plant skin.

A cuticle is found over every young aerial plant surface, coating even the apical tip beneath repeated layers of leaf primordia. It penetrates down into the sub-stomatal cavity, under the superficial leaf salt glands in salt-secreting plants and covers the enzyme-secreting glands of insectivorous plants. It reaches its most massive development in the thick, commercially-valuable wax layers on the leaves of the carnauba palm, *Copernicia cerifera*. The epicuticular wax of such a plant surface was man's first plastic. It reaches its peak of subtlety in the delicate detachable wax scales of the trap mechanism of the *Nepenthes* pitcher from which no arthropod can climb. In its routine role it protects the plant from desiccation, hinders the penetration of pathogen and insect and may reflect, diffuse or concentrate the rays of the sun.

Under intensive agriculture, more and more substances, damaging and beneficial, are thrown or fall on to leaf surfaces. Amongst the former are weed-killers and the fall-out from industrial and nuclear activity; amongst the latter are insecticides, foliar fertilizers and irrigation water. Recently it has become apparent that almost every plant surface not only absorbs materials, but also discharges them. In fact the whole plant's surface, from apical tip to root cap, is an oozing, flaking layer. Sometimes, as in the root cap, this appears to be a highly organized and purposeful exercise. In others, such as most leaves, it is not at all clear how the plant benefits from this loss, but an often vigorous epiphyllous flora and fauna obviously does. Where the animal carries its alien flora, generally of a beneficial kind, on the moist warm walls of its gut, the plant does likewise, but on the exposed surfaces of root and shoot.

Many leaves, through leaching, are rich sources of nitrogenous compounds, polyphenols and salt solutions. They may discharge, into the atmosphere, heavy metals concentrated from the soil and also a range of hydrocarbons. Much of the coal and oil beneath the earth's surface may derive from this source. Young root

surfaces may discharge into the soil a range of polysaccharides and some proteins, some of which may be signalling compounds to parasites or symbionts.

Any plant surface may become the home of a wide range of parasites or symbiotic organisms. Some epiphyllous plant species may have no other home than the surface of a tropical leaf and nitrogen-fixing micro-organisms on leaf or root are widespread.

The plant surface too is the recognition layer for friend or foe. It is the benevolent and compatible surface of the pollen grain or graft interface or the hostile surface of fungal hypha or *Cuscuta* haustoria.

From the inert, enveloping skin of the eighteenth century the plant surface is now seen as a dynamic adaptable envelope, flexible in both the import and export of materials, forming both an ecosystem in its own right and the first barrier between the moist, concentrated, balanced cell and a hostile ever-changing environment.

Oxford and Edinburgh, 1983 B.E.J. and C.E.J.

Contents

1 The Historical Perspective

'The structure of leaves is very simple. It consists of an outer skin or cuticle which is full of pores, the upper surface being varnished as it were. Cellular tissue is seen when the cuticle is removed' from *The Library of Agricultural and Horticultural Knowledge*, BAXTER, J(1834). Lewes.

Over 300 years ago Robert Hooke, who first used the word 'cell' in a description of plants, was also the first to describe in detail the leaf surface and the hairs on a nettle (*Urtica dioica*).The presence of a superficial non-cellular membrane was first suggested by Ludwig in 1757 and Brongniart (1830–34) isolated this membrane which he named the 'cuticle' (Fig. 7-5), by allowing a cabbage (*Brassica*) to rot in water. Henslow (1831) detached a similar skin with nitric acid from the corolla, stamens and style of the foxglove (*Digitalis purpurea*) and Brodie in 1842 detected cuticles in fossil plants. The mid nineteenth century was a period of vigorous activity into the physiology of leaves and 'cuticular' versus 'stomatal' transport of gases was fiercely debated. Darwin was aware of the permeability of the cuticle over the glands of insectivorous plants (1875) and it was apparent as early as 1845 that this membrane was not only external but lay within, for example, the sub-stomatal chamber (von Mohl).

Ideas that are still the subject of debate have long histories. Could the plant surface wax pass out through the cuticle in a solvent carrier? This was suggested by Wiesner as early as 1871. De Bary (1884), in a monumental work on the classification of plant surface waxes, proposed that they migrated through cuticular canals; the suggestion is often repeated in modern literature. Apart from De Bary's description of almost every known waxy plant surface (transmission and scanning electron microscopy have only added detail to his major work), he also investigated commercial waxes, including carnauba (see section 7.7) and experimented both on their chemistry and biosynthesis. Lee and Priestley (1924) brought the subject well into the twentieth century with widespread investigations into the role of the environment in the development of the cuticle and its components, its biosynthesis, physiological functions and ontogeny. Chibnall and Piper's pioneering and very extensive work on the chemistry of plant waxes in the late 1920s and 1930s, laid not only a groundwork for the major typing and distribution of plant waxes but also established many of the basic ideas of their biosynthesis. The ground was now prepared for the refined techniques of the post-war years, chromatography in all its forms, autoradiography and electron microscopy, to exploit and to illuminate.

2 Techniques for the Study of Plant Surfaces

A vast spectrum of techniques, ranging from the most simple and observational to those involving sophisticated apparatus, has been applied to the study of the plant surface. Most of these are routine and have broad application elsewhere and have likewise been described in detail elsewhere. Only a few, e.g. scanning electron microscopy and certain isolation and analytical methods for chemical components are given a relatively full treatment here, the others are dealt with briefly and reference is made in the reading list to a more extensive coverage.

2.1 Microscopical Methods

2.1.1 Polarized light microscopy

Plane-polarized incident light can show how large molecules are orientated within a structure. If the molecules are non-random then plane-polarized light will distinguish between the axes of the substance; its refractive index will differ according to which axis is measured. The early anatomists distinguished in this way between the isotropic, randomly orientated layers, e.g. the pectin and wax layers, and the anisotropic orientated layers, e.g. the cutinized layer and cellulose-rich wall layers of a plant's epidermis.

2.1.2 Staining for the light microscope

Conventional staining for the light microscope has added little to our knowledge of the structure of plant surfaces principally because of the lack of a precise reaction between, for example, the stains for pectin (ruthenium red is specific only for carboxyl groups) or phloroglucinol in HCl which does not clearly distinguish between suberin and cutin. The waxes of plants are chemically so diverse that no useful staining reaction exists for them *in situ*. But separated waxes may be selectively stained (see section 2.2.2).

2.1.3 Scanning electron microscopy (SEM)

SEM is the simplest and most direct method for high resolution microscopy of plant surfaces. The most usual operating mode, the emissive mode, provides chiefly topographical information, producing an image with an uncannily natural appearance. The effect is similar to a reflected light micrograph or macro-photograph, but with about 300 times the depth of focus obtainable from light images.

A modern SEM has an optimum resolution of about 10 nm with biological specimens compared to about 2 nm for transmission electron microscopy (TEM) and 250 nm for optical microscopy (Grimstone, 1976).

The SEM may be used to examine the surface of any small (<1 cm^3), dry, robust specimen. Non-conducting materials tend to charge up in the electron beam, causing beam deflections and image distortion. This can be overcome by coating the specimen with a conducting film of carbon and/or gold or gold/palladium alloy, techniques borrowed directly from TEM, which conduct charge to earth via the specimen stage.

Delicate plant specimens, such as primary roots which collapse under vacuum, are usually dehydrated by critical point drying (CPD). The specimens are first fixed and dehydrated in a water/alcohol series and the alcohol replaced with acetone, amyl acetate or Freon 113. The solvent is then in turn replaced with liquid carbon dioxide in a pressure bomb. Finally the pressure bomb is sealed and its temperature raised to the 'critical point' (31°C), at which carbon dioxide undergoes a change of state, from liquid to gas, at the *same* volume. The gaseous carbon dioxide may be bled off and the specimen is thereby dried without having passed through a gas/liquid interface.

CPD gives excellent results with many tissues, but the solvents used may destroy plant epicuticular waxes. Better results are often obtained if the surfaces of plants are mounted fresh as untreated tissues and examined immediately using a low voltage beam to minimize charging, or briefly coated with gold in a sputtering device. Rapid handling and observation are essential and specimens cannot be stored. Delicate tissues, such as rose petals, which must be examined in an untreated state can be frozen in liquid nitrogen and viewed, still frozen, on a cryogenic specimen stage. This method, though technically demanding, provides a very high standard of tissue preservation free from artefacts of dehydration and solvents (Fig. 4-3).

Dry plant materials, seeds, pollen or wood which present no special problems of dehydration may be examined coated or uncoated as desired.

The SEM beam causes X-rays to be emitted from the specimen which are characteristic of the element which emits them, and an image can be constructed by an X-ray spectrometer of the distribution of an element in the scanned area of the specimen. X-ray emission mode microscopy has been used to investigate the chalk-secreting glands of *Plumbago capensis* and the deposition of silica in the tissues of *Equisetum*, rice (*Oryza sativa*) and other cereals. The cathodoluminescent mode can be used to visualize the distribution of fluorochromes which emit light in the electron beam. Various modes can be used in combination to give different types of information about the same area of the specimen surface. Because the SEM image is constructed from a linear sequence of information it is ideally suited to digitization and image processing. At the simplest level this permits electronic control of the image quality – contrast, and edge definition. More sophisticated analysis enables objects to be identified, counted and measured and it is possible to record the image, using video recorders, for subsequent computer analysis.

2.1.4 Transmission electron microscopy (TEM)

TEM made its first major impact on the study of the plant surface through the indirect replica method (Fig. 2-1) (Juniper and Bradley, 1958). This technique permits almost any surface, however delicate or opaque to be examined. Carbon, the commonest replica material, has virtues of coherence, low electron contrast,

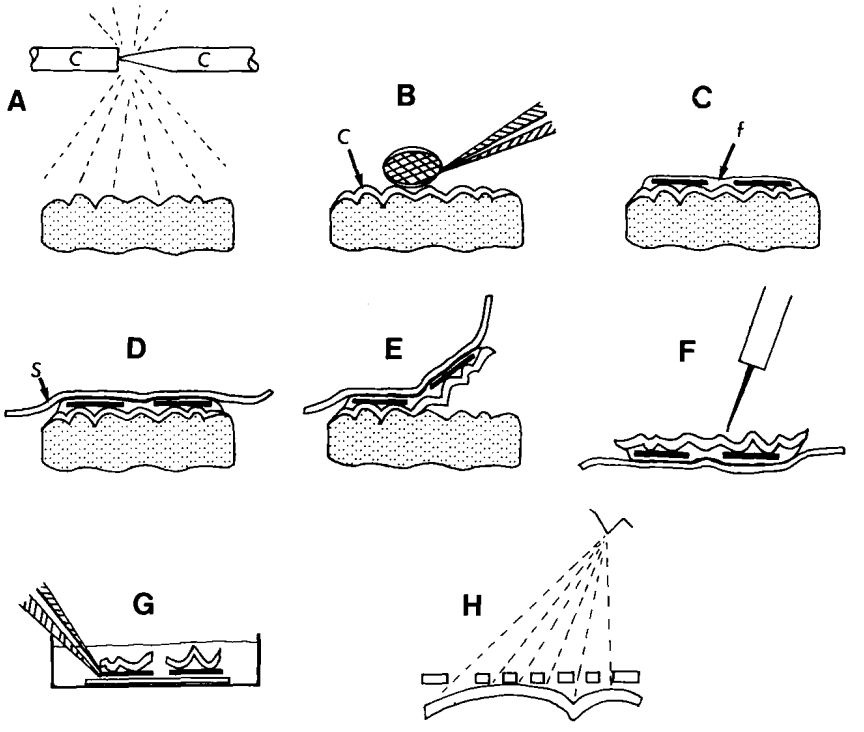

Fig.2-1 The carbon-replica technique. **A** The fresh plant surface is coated, in a vacuum, with evaporated carbon from an arc (C). **B** Grids dipped in Formvar are placed on the carbon-coated surface, which is then (stage **C**) flooded with 2% Formvar in chloroform (F). **D** As soon as this is dry a layer of sellotape (S) is placed over the Formvar layer, mechanically stripped (stage **E**) and the perimeter of each grid (stage **F**) is scored around with a needle. **G** This four-layered sandwich is placed in a bath of chloroform to remove the Formvar, the carbon replica is lifted away on the grid, inverted and obliquely shadowed, through the grid bars (stage **H**), with an evaporated heavy metal such as platinum.

conductance, the ability to replicate re-entrant angles and to replicate biologically moist surfaces. A good carbon replica (Figs 2-2, 2-3 and 3-2) can resolve 2 nm and combined, as here, with metal-shadowing reveals a wealth of structure well below the resolution of the light microscope. However, the techniques are tedious and suffer from the disadvantage present in all TEM work that the third dimension is suppressed or almost eliminated. It has, therefore, for almost all problems been supplanted by the SEM (Figs. 2-2, 3-7, 4-3, 4-7 and 5-2) (Baker and Holloway, 1971, Parsons *et al.*, 1974).

Difficulties of adjusting embedding materials to cope with the preservation of structures as diverse as cellulose, cutin and wax delayed the application of ultra-thin sectioning to plant surface studies. Many of these difficulties have now been overcome and the whole battery of minor techniques associated with TEM, including negative staining, freeze-etching, radioactive and inert X-ray analysis, ultra-histochemistry and high-voltage TEM, are now used.

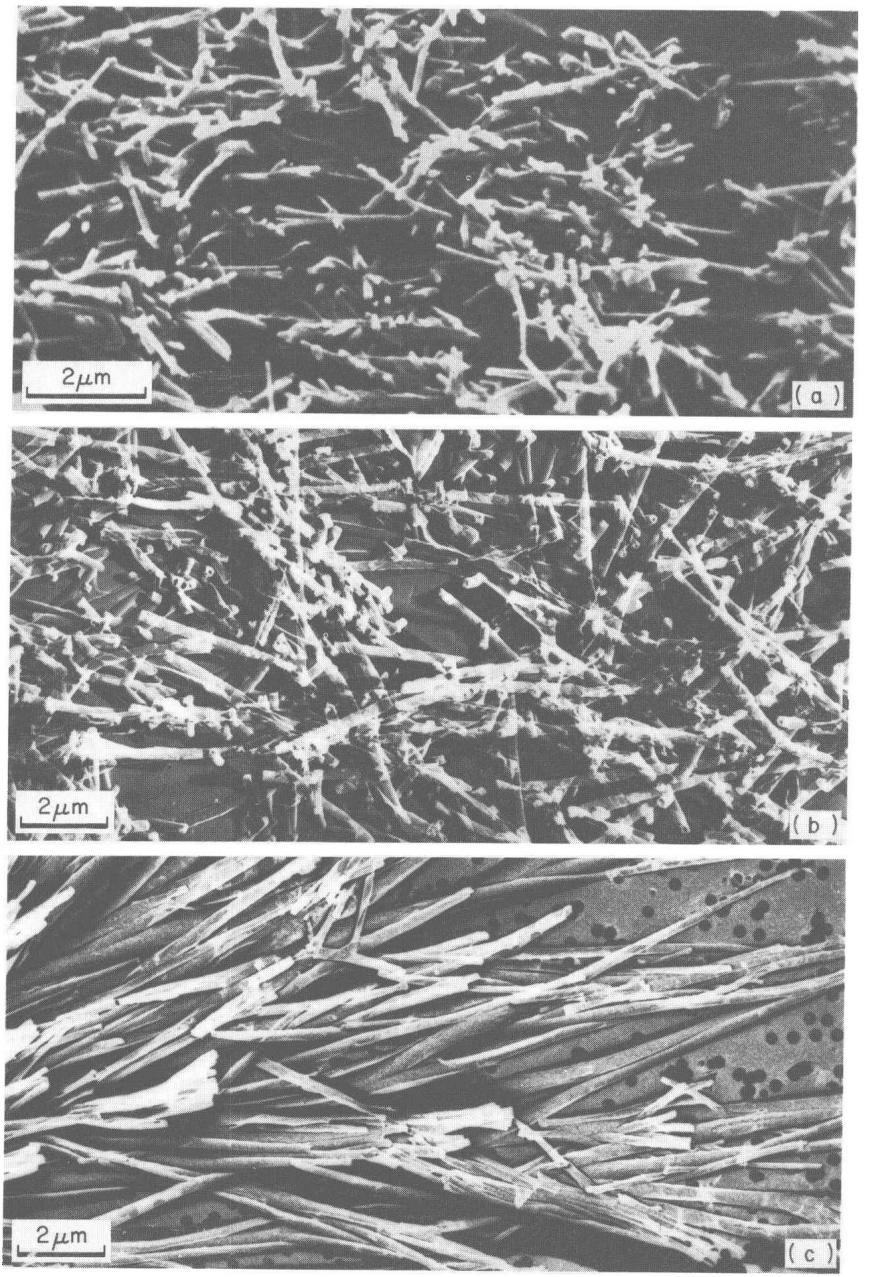

Fig. 2-2 Three views of the same surface. **A** SEM of Sitka spruce (*Picea sitchensis*) leaf surface showing epicuticular wax tubes. **B** As above, but TEM platinum-shadowed carbon replica. **C** Sitka spruce wax crystals washed from the leaf surface and recrystallized *in vitro* from solution in hexane. Gold /palladium shadowed carbon replica. TEM.

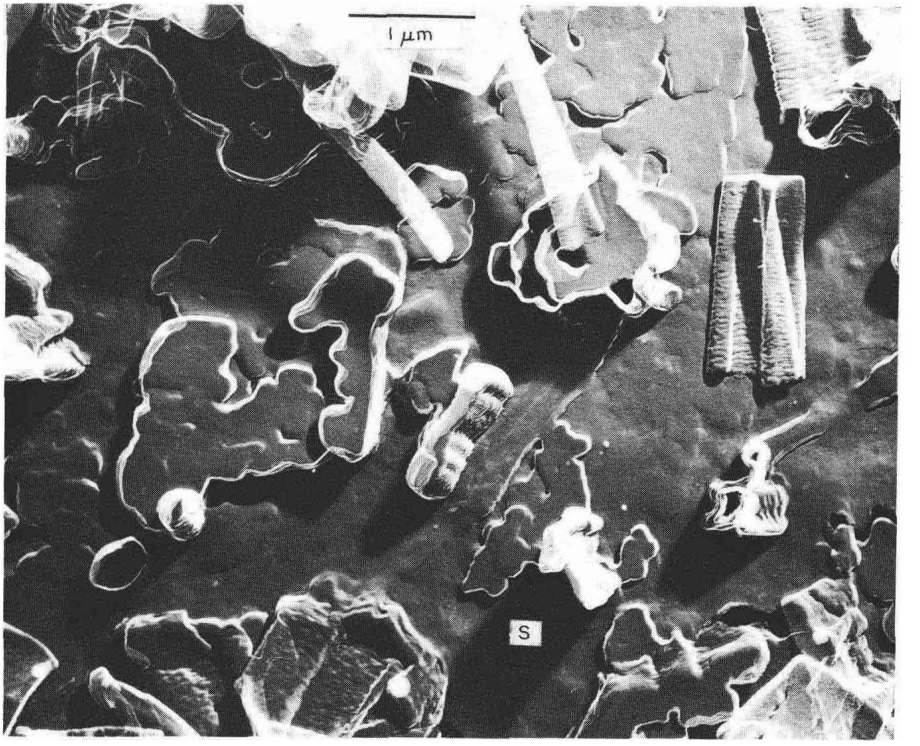

Fig. 2-3 A platinum-shadowed carbon replica of a cabbage (*Brassica oleracea*) leaf surface. (S) is the 'shadow' formed by the exclusion of platinum from the carbon surface.

2.2 Chemical Methods

2.2.1 Isolation of the epicuticular waxes

Plant surface waxes are chiefly composed of long chain aliphatic hydrocarbons with chain lengths between C20 and C35. The molecules vary in polarity depending on the position and kind of any substituted groups present, e.g. carboxyl (−COOH), hydroxyl (−OH) (see Figs 2-4 and 7-3). Most of these compounds are readily soluble in organic solvents. The non-polar *n*-alkanes dissolve easily in non-polar solvents such as hexane or benzene, but are much less soluble in polar solvents such as acetates and alcohols. The converse is true with the very polar carboxylic acids.

Since plant waxes contain a mixture of polar and non-polar constituents, solvents must be carefully chosen if they are to dissolve a representative sample of the wax constituents from the plant surface. Chloroform appears to introduce the least selective bias and it is the most generally used solvent for isolation of plant epicuticular waxes.

Most of the epicuticular wax dissolves from plant surfaces in the first 10 to 30 seconds of immersion in chloroform at 20°C. Successive brief washings in fresh solvent remove cuticular wax constituents from progressively deeper locations in the cuticle. These differ qualitatively from the epicuticular wax, but there are

many compounds in common. Continued solvent extraction removes more of the lipid constituents of cells and fewer cuticular lipids, and for this reason intact, undamaged leaves are used. The wax can be extracted from separate surfaces of a broad leaf by washing them separately in a stream of chloroform from a burette. More local wax extraction can be achieved by swabbing plant surfaces with cotton swabs soaked in chloroform.

When sufficient wax is present it can be removed physically by scraping, or wiping with dry cotton swabs. In exceptional cases the wax layer may be thick enough to remove with tweezers, as in leaves of the carnauba palm (*Copernicia cerifera*). Useful information about the distribution of wax constituents in the epicuticular and cuticular layers comes from the comparison of wax obtained by physical means with samples obtained by different methods of solvent extraction.

2.2.2 Thin layer chromatography (TLC)

Early analytical methods needed long and tedious chemical procedures and large amounts of material for separating waxes into their constituents. Modern methods rely on chromatography for separation of the constituents and on sensitive methods which permit unequivocal identification of trace amounts of individual compounds (Holloway and Challen, 1966).

Thin layer chromatography (TCL) is a simple and sensitive method of separating wax constituents by constituent class. In this method a thin layer (0.25 mm) of absorbent, usually silica-gel, is spread on a glass plate. Spots of wax solution are applied to the surface of the plate with a glass capillary, and a solvent front is allowed to ascend the plate from one edge, carrying wax compounds with it. The distance travelled by a compound depends on its polarity and the polarity of the solvent. Chloroform solubilizes wax constituents so uniformly that most are carried to the solvent front. The least polar constituents are best separated with pure benzene, (Fig. 2-4), and the more polar constituents are resolved by a 60/40 mixture of chloroform and ethyl acetate. Details of staining reactions and applications of TLC for natural waxes are described by Holloway and Challen (1966).

2.2.3 Gas–liquid chromatography (GLC) and mass spectrometry (MS)

GLC is a powerful analytical method, enabling the general classes of wax compounds to be further resolved into their individual homologues and enabling quantitative estimations to be made. In GLC the stationary phase could be a hydrocarbon oil or silicone film on the surface of a powdered inert solid such as firebrick or diatomaceous earth, packed into a stainless steel column. The mobile phase is an inert gas, usually nitrogen or helium. The minimum quantity of a compound detectable using this system is about 0.01 μg.

Comparison of the behaviour of unknown compounds with known standards gives much circumstantial evidence of the identity of compounds, particularly when coupled with information about staining reactions from TLC. Such evidence can be further reinforced by derivatization of a compound which often results in characteristic shifts in retention and by information from infra-red absorption spectroscopy. However, only mass spectrometry (MS) is capable of unequivocal identification of compounds and it is used as the final arbiter. As a compound is eluted from the GLC column it passes into the MS where it is ionized by an electron beam. The molecules fragment into characteristic

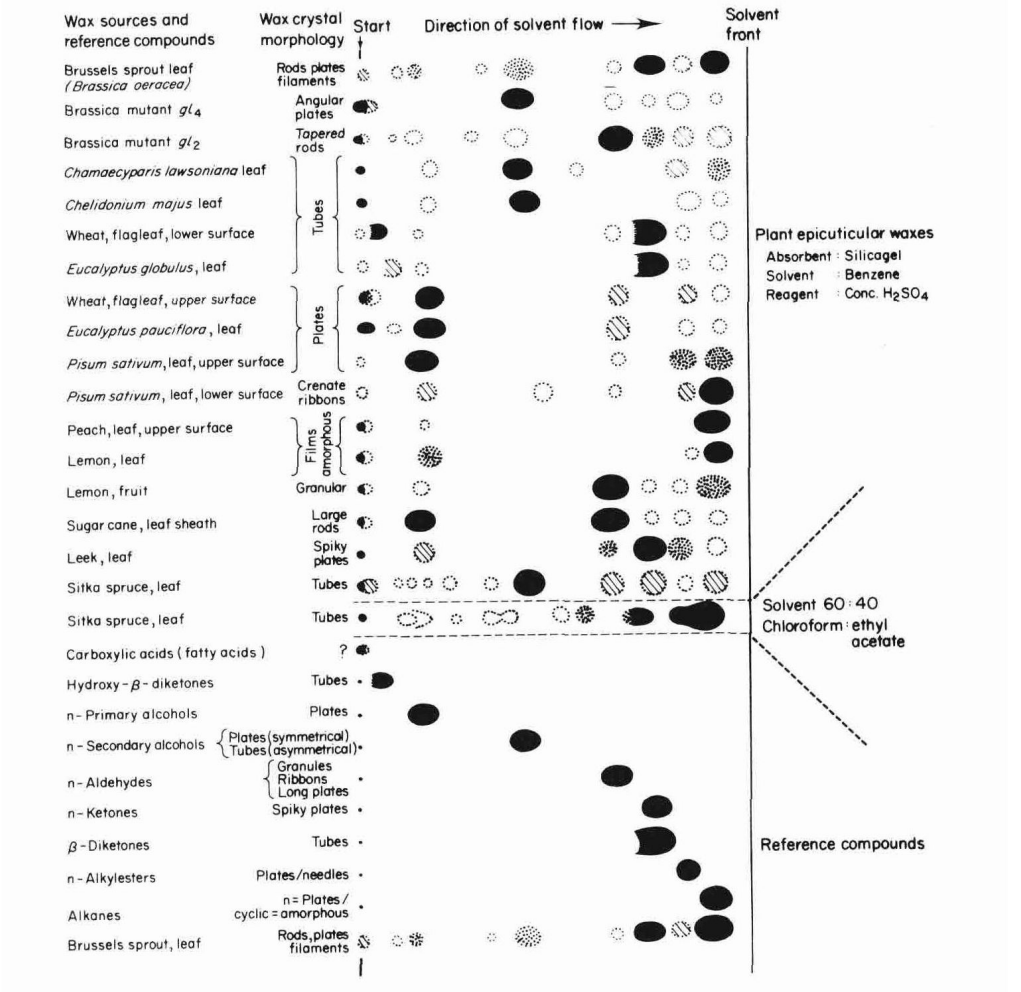

Fig. 2-4 Thin layer chromatogram of plant epicuticular waxes compared with known standards and correlated with wax crystal morphology.

patterns of ions, often including the parent ion, the positively-charged parent molecule. This ion 'fingerprint' can identify and quantify mixtures of isomers which are inseparable using GLC alone.

2.2.4 Isolation of plant cuticular membranes

The cuticle is strongly resistant to chemical attack and may survive natural decay long after the cells have gone, particularly in acid anaerobic environments. Early workers found that they could isolate plant cuticles by treatments as diverse as digestion in dilute nitric acid and allowing leaves to rot

in water (Fig. 7-5). More recently cuticles have been isolated with mixtures of nitric and chromic acids, concentrated sulphuric acid and saturated solutions of zinc chloride in hydrochloric acid (Holloway and Baker, 1968).

Gentler methods are used for critical analytical work and it is now common practice to use enzymes. Mixtures of cellulase and pectinase (2–4%, pH 4.0, 37°C) remove the cell walls and detach the cuticle by dissolving the pectin (Fig. 3-4) which bonds it to the epidermis. Similar results are obtained with enzymes from *Helix pomatia* gut, or by incubation in the rumen of fistulated animals.

Mixtures of 2% ammonium oxalate in 0.5% oxalic acid, solutions of hydrogen peroxide, and EDTA have also been used and yield cuticles of the same weights as those obtained with enzymes.

The cuticles of many species resist isolation using these gentler methods and *Fragaria* cuticles fragment badly making them difficult to handle and measure. Holloway and Baker (1968) found that in difficult cases they could still isolate cuticular membranes with zinc chloride/hydrochloric acid mixtures. Conifer cuticles resist any of these methods, although they are isolated naturally by fungi and arthropods in leaf litter, and can be found there in considerable quantities.

3 The Anatomy, Ultrastructure and Biosynthesis of the Plant Surface

3.1 Lower Plant Surfaces

Unicellular algae, unlike the protozoa, have a wall of cellulose and pectin. As these algae evolved they aggregated into colonies embedded in mucilage, a chemical extension of the wall.

A seaweed is a relatively massive accumulation of cells with a thallus many cells thick, but it still has this mucilaginous coating. The only permanent slimy layer in a higher plant is that of the root cap which, incidentally, in maize has fucose as one of its mucilage sugars. This slime layer holds large quantities of water, resists loss by evaporation and assists the algal invasion of the littoral zone between the tide marks, where the plants are exposed twice daily to dehydration. Its lubrication also reduces the damage by the rubbing of one plant on another.

The seaweeds, except those briefly above high tide, have no drought problem, and do not hinder the movement of water through the plant surface. But land plants, particularly those in the drier habitats, have to conserve water all the time. In the higher plants the gas exchange surface is not, as in the algae the outside, but the mesophyll cell surfaces, loosely-packed photosynthetic cells with air spaces between and gas diffusion around them. The mesophyll is covered by the epidermal cells which are in turn coated with the water-impermeable fatty cuticle, separating the mesophyll from environmental contact. The light-transparent epidermal cells are interrupted by the stomata. These act as ventilators and allow the mesophyll cells to photosynthesize in a bright environment under controlled conditions of humidity and CO_2 concentration.

The evolution of this arrangement is obscure, but in the liverworts such as the Marchantiales, there are large, permanently open pores leading to internal photosynthetic tissues (Fig. 3-1), which may represent an intermediate state. A similar arrangement is known in fossils of primitive vascular plants such as *Spongiophyton*. The cuticle too plays an important part in the evolution and differentiation of land plants. It is the final barrier to water loss and must have been an early development in evolution as the thalloid layer of the early plant form began to develop and thrust up into the desiccating wind.

A cuticle is always present in land plants. However, the vestigial, but occasionally waxy cuticle in most Bryophytes – moss leaves are usually only one cell thick – restricts them to wet habitats, or has brought about a tolerance to desiccation without permanent damage. A fern, intermediate between higher plants and Bryophytes in its degree of terrestrial adaptation, has a prothallus (gametophyte generation) lacking either epidermis or cuticle, thus restricting it to a moist habitat. The sporophyte generation however is vascular, has a