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The use of the scanning reflection electron microscope in the study of plant and microbial material

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SYNOPSIS

The scanning reflection electron microscope has been used over the past two years to investigate the surface topology of a wide variety of plant and microbial material. The comparative ease of specimen preparation, coupled with the greatly increased depth of focus and resolution over what is available in light microscopy, has made it possible to resolve surface features as small as 200 Å on pollen grains, seeds, and a wide range of algæ. Robust specimens, such as many pollen grains and diatoms require very little preparation other than air drying in a dust free atmosphere. More labile specimens require either chemical fixation and dehydration, or freeze drying in order to preserve finer details of their surface. The various preparative techniques are described in detail. Some success has been achieved in the examination of material embedded in either paraffin wax or resin, sectioned and the embedding medium subsequently removed before being placed in the microscope. By combining the information obtained by scanning reflection electron microscopy with data from transmission electron microscopy it has been possible to reconstruct the structure and surface topology of several pollen-grain walls.

INTRODUCTION

The advent of the scanning reflection electron microscope has introduced a valuable new tool for studying cellular sub-structure and the instrument has been used over the past two years to investigate the surface topography of a wide variety of plant and microbial material. In the instrument a beam of electrons is focused to a fine point on the specimen and made to sweep or scan the surface of the specimen in a "raster" as in a television system. The electrons which strike or slightly penetrate the surface cause "secondary" electrons to escape from the specimen. These secondary electrons, the number and energy of which are governed largely by the character of the surface being examined, are collected and amplified. The resulting signal is used to control the brightness of another focused beam of electrons which are made to sweep across the screen of a cathode ray tube coated with a long-persistence phosphor in synchrony with the beam scanning the surface of the specimen. The resulting image has a marked three dimensional character, which may be recorded from a separate display tube with a short-persistence phosphor, either on Polaroid or 35 mm film.

Specimens having complex shapes and uneven surfaces can be examined at high magnifications and the instrument will resolve objects as small as 150 Å—more than

ten times the resolving power of the optical microscope. Even at the same magnification the depth of focus of the scanning reflection electron microscope is several hundred times greater than that utilizable in optical and transmission electron microscopes. For a more detailed description of the instrumentation of the scanning reflection electron microscope, the reader is referred to the articles and reviews by Oatley *et al.* (1965), Thornton (1965), and Oatley (1966).

The amount of energy dissipated by the specimen is very small compared with that produced by an electron transmission beam, and this allows fragile objects such as whiskered and hairy growth and fine textile fibres to be examined in the instrument. A number of studies have also been made on various types of biological material. Although the amount of specimen preparation is minimal when compared with the elaborate and extended techniques used in transmission electron microscopy, certain precautions are necessary to ensure optimal preservation.

At the time this paper was prepared there were two different types of scanning reflection electron microscopes which were available commercially. The Japan Electron Optics Laboratory Co. Ltd. manufacture the JSM Scanning Type Electron Microscope which has a resolving power of 500 Å and magnification range of between 100 and 100,000 diameters. The Cambridge Instrument Company, England manufacture the Stereoscan Mk IIa scanning reflection microscope, having a resolving power of 150–300 Å and a magnification range of between 14 and 200,000 diameters. The author has had the opportunity of working with both types of machine, though the findings presented in this paper are entirely the result of work carried out on the Stereoscan Scanning Reflection Electron microscope.

METHODS AND RESULTS

The description given below is of methods which have been tried and evaluated over a period of two years on a wide variety of plant and microbial material. Various methods have been applied to different materials and, although the techniques which are described under the various groupings have been found to give optimal preservation, it is suggested that a number of different methods should be applied to any new material. The major problem in preserving material for the scanning reflection electron microscope is the removal of water from the specimen with the minimum of tissue damage occurring during the process (Fig. 19).

Robust dry specimens (Figs. 1–6)

This group includes pollen grains, seeds, spores, wood, and bark. They are referred to as robust and dry specimens as they can be placed into the microscope with a minimum of preparative technique. It is necessary to securely attach the sample to the specimen stub, and the most satisfactory method has been to use a glue like Durafix, or the glue obtained from dissolving the adhesive from Sellotape with chloroform. The specimen stub is lightly coated with the glue, and the sample sprinkled on the surface when the adhesive becomes tacky. In the case of pollen it it necessary only to tap the dehisced anther to ensure that sufficient pollen falls on the sticky surface. Large specimens such as seeds, may be arranged individually to ensure that a number of different faces are exposed.

It is now necessary to ensure that any moisture remaining in the specimens is removed without causing disruption of the surface. In the case of these robust specimens, it is usually only necessary to place them in a dust-free atmosphere and allow them to dry slowly over a period of 48 hours. The drying may be completed by placing the specimens over some chemical desiccant such as silica gel, or phosphorous pentoxide. This technique will usually give good results with many free

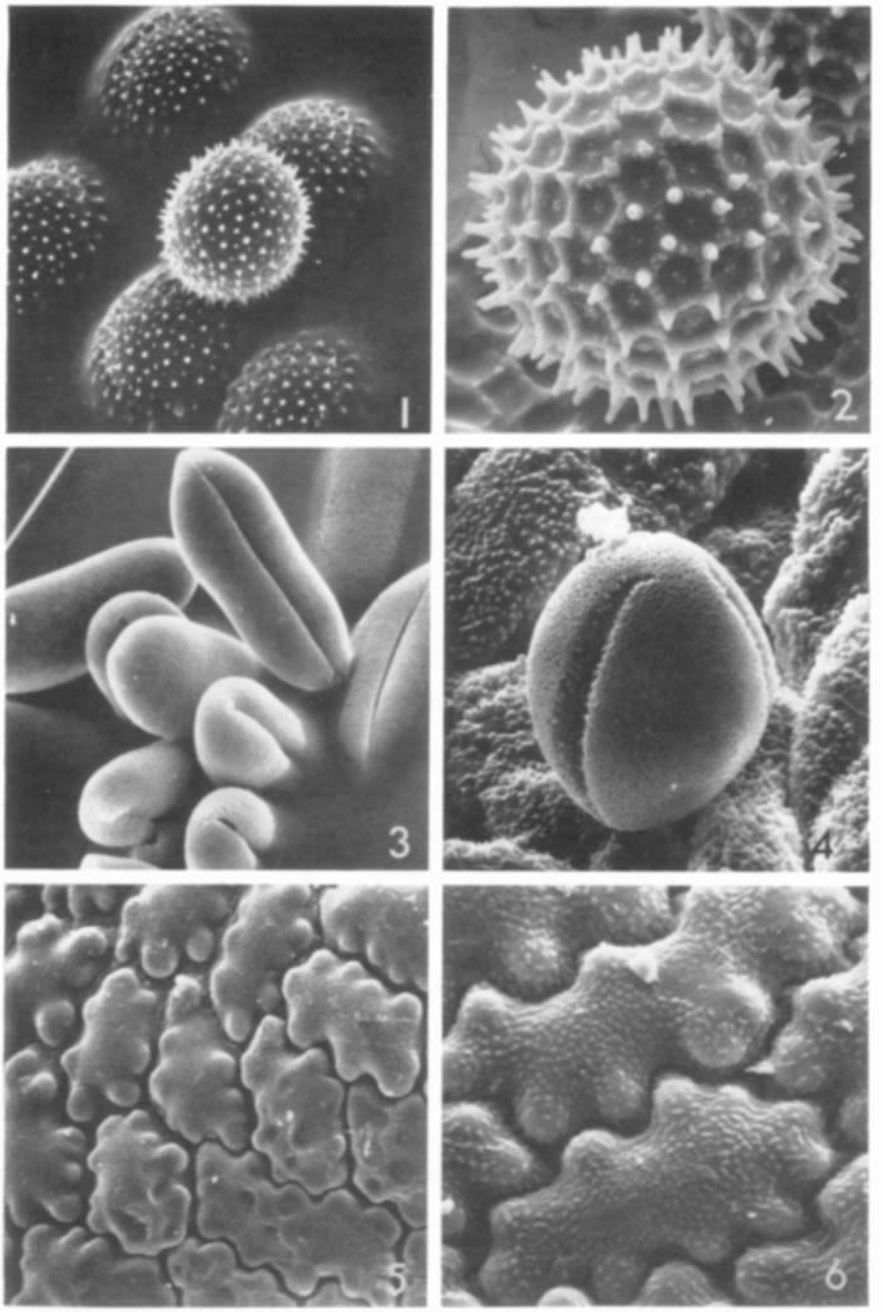

Fig. 1. Pollen grains of *Malva sylvestris*, × 240.
Fig. 2. Pollen grain of *Ipomœa purpurea*, × 720.
Fig. 3. Pollen grains of *Sisyrinchium bermudianum*, × 670.
Fig. 4. Pollen grain and tapetal cells of *Helleborus fœtidus*, × 460.
Fig. 5. Surface of the seed of *Arenaria ciliata* subsp. *hibernica*, × 370.
Fig. 6. Surface of the seed of *Arenaria ciliata* subsp. *pseudofrigida*, × 615.

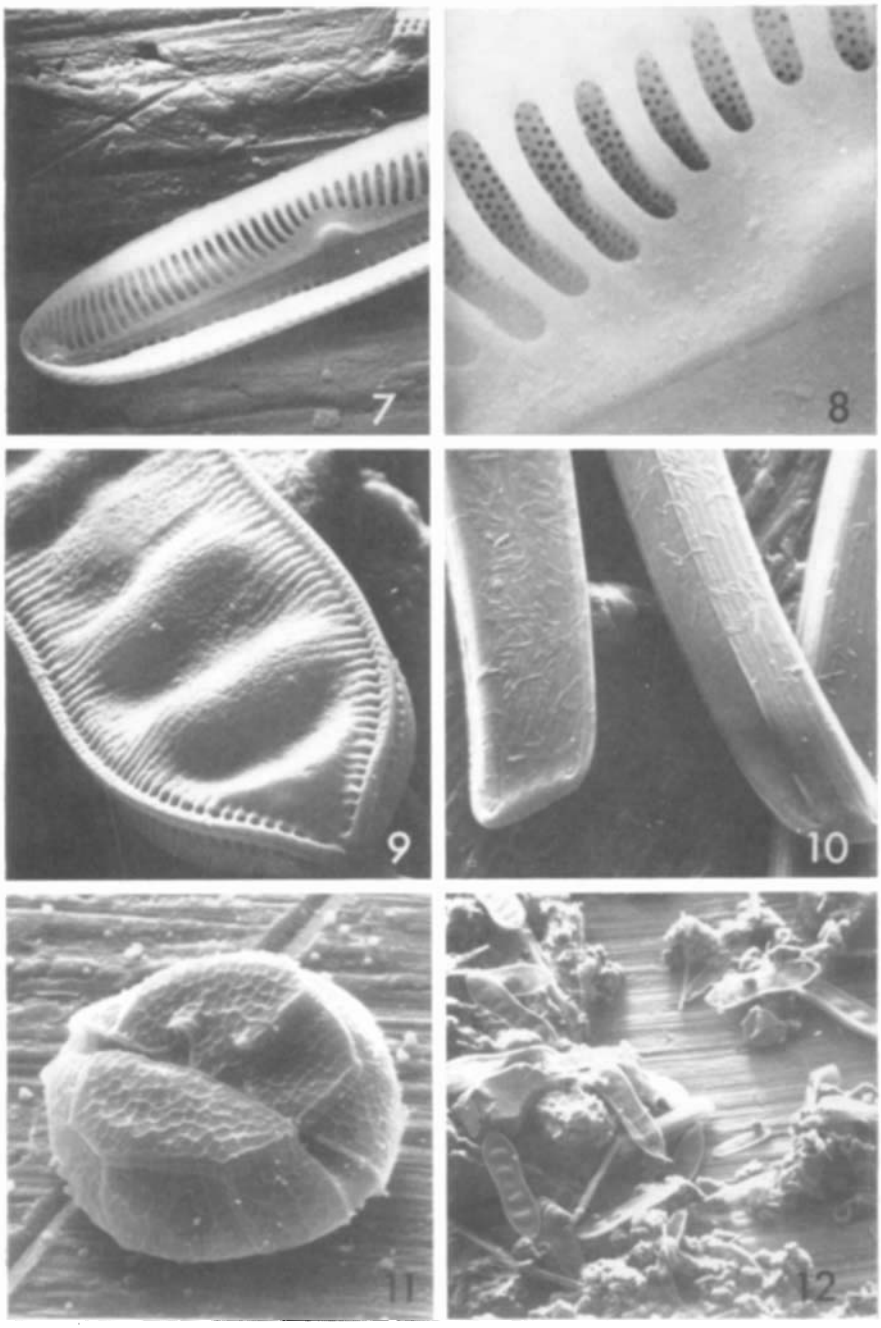

Fig. 7. Cleaned frustule of the diatom *Pinnularia* sp. × 1550.
Fig. 8. Detail of the diatom *Pinnularia* sp., × 7800.
Fig. 9. Cleaned diatom frustule, × 1550.
Fig. 10. Fresh diatoms (*Eunotia* sp.) coated with bacteria, × 370.
Fig. 11. The dinoflagellate *Peridinium* sp. showing cellulose plates, × 1050.
Fig. 12. Cleaned diatom frustules, × 160.

pollen grains, nearly all herbarium material, and most specimens of wood and bark.

Robust wet specimens (Figs. 7–12)

There is little difference between this group and the previous one, except that the specimens are usually suspended in liquid. In most cases of this type it is possible to dispense with the glue, and the sample may be placed directly onto the specimen stub and the liquid allowed to dry. It has been found in the case of living specimens that there are biological polymers such as mucopolysaccharides or polysaccharides on the surface of the material being examined which attach it more or less firmly to the specimen stub. As a corollary to this, it has frequently been found that bacterial and algal specimens which have a thick sheath and capsules not only adhere more firmly but dry more slowly and are better preserved. As before, the specimen is dried slowly in a dust-free atmosphere, and the last traces of moisture removed using vacuum drying or chemical desiccants.

Labile specimens

Unfortunately the majority of plant and microbial material is very susceptible to the drying procedures previously outlined, and it is frequently necessary to resort to gentler methods of specimen preparation. Some success has been obtained with some of the smaller algæ and microbial material by adding a drop or two of 1% osmium tetroxide to the culture or sample of the specimen, allowing it to stand for a few minutes, and then thoroughly washing the specimen free of osmium by repeated centrifugation and irrigation. The specimen may then be dehydrated through a graded ethanol or acetone series, and once in the 100% solution, may then be treated as a robust specimen. In some cases it has been possible to dispense with the osmium fixation.

Freeze drying (Figs. 13–18)

This has proved to be the best method for preparing labile material for examination in the scanning reflection electron microscope. Only living specimens have been examined by this method. A small amount of the organism is placed on the surface of small copper discs. The discs, which are 1·5 mm thick and 5 mm in diameter, are smooth and highly polished on both sides. One of the two sides is slightly etched with dilute nitric acid, and the discs are thoroughly cleaned in an ultrasonic cleaner. The smooth side ensures that good thermal contact is made between the disc and any conducting surface. The other side is etched slightly to ensure that the specimen will readily affix to the surface.

It has been found that small organisms such as unicellular algæ, bacteria and yeasts are best applied to the copper disc in the form of an aerosol, while other specimens are placed as a thin layer onto the surface, and the excess water gently removed with filter paper. In the case of thin-walled pollen grains and spores, which are not sufficiently robust to withstand the previous treatments, it is found necessary to apply a thin layer of Sellotape glue to the surface of the copper disc, and sprinkle on the specimen as before.

As soon as the specimen has been applied to the surface it is immediately plunged into either iso-pentane or Freon 13 cooled with liquid nitrogen, or directly into liquid nitrogen at −158°C. This instantly freezes the specimen and the copper discs may remain in the coolant until the next stage of the procedure is ready. In the initial experiments with freeze drying, the copper discs were plunged into the liquid nitrogen and placed specimen side up on the surface of a large block of polished brass, previously cooled and placed below the surface of the coolant. The

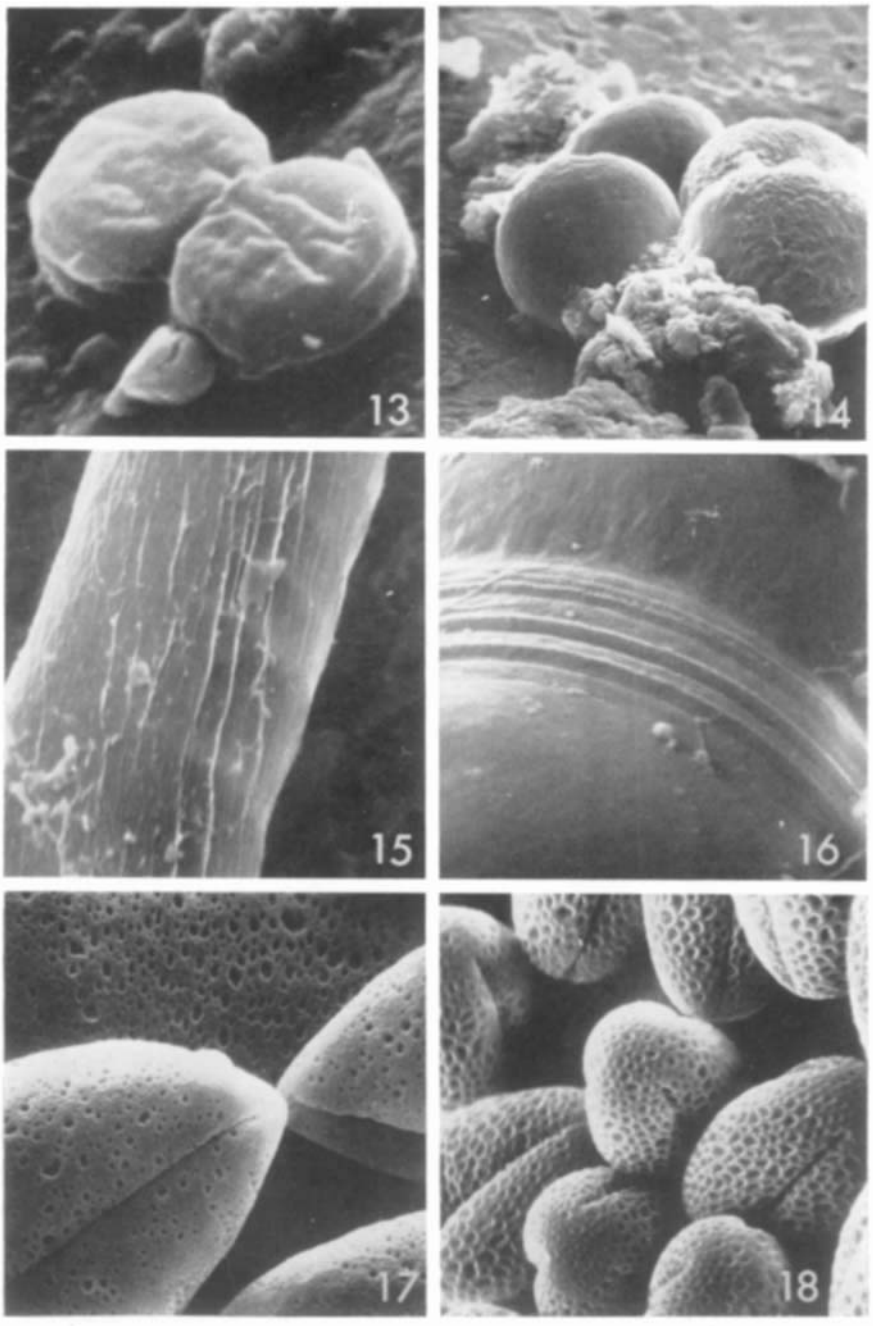

Fig. 13. The unicellular blue-green alga *Anacystis montana*, × 8900.
Fig. 14. The unicellular blue-green alga *Chroococcus turgidus*, × 680.
Fig. 15. The filamentous blue-green alga, *Lyngbya majuscula*, × 2650.
Fig. 16. The filamentous green alga *Oedogonium cardiacum*, × 5200.
Fig. 17. Pollen grains of *Endymion non-scriptus*, × 2100.
Fig. 18. Pollen grains of *Cheiranthus* sp., × 1350.

block and copper discs were then quickly transferred to a small vacuum container in a litre Dewar flask cooled with a solid CO_2 and acetone mixture and fitted with a phosphorous pentoxide vapour trap. The container was evacuated to a pressure equivalent to 10^{-3} torr and continuously pumped overnight at the dry ice and acetone temperature (approx. $-70°C$). After this initial period, the vacuum was maintained and no more dry ice was added to the cooling mixture. This caused the whole apparatus to come slowly to room temperature. The specimens were evacuated for a further two hours at room temperature, after which dry air was emitted to the vacuum chamber. The copper discs were removed and placed in a desiccator. The principle virtue of this method was that it required a minimum of expense and apparatus to construct, although it gave very variable results, due mainly to the formation of ice crystals during the evacuation procedure at $-70°C$.

More success was obtained using the Pease Tissue Dryer, manufactured by Edwards High Vacuum Ltd., to remove the water from the specimens. The specimens were placed on the copper discs and rapidly frozen as described previously. The discs were then quickly transferred to the thermo-electrically cooled platen of the Tissue Dryer. Accurate control of the specimen temperature was achieved by two thermo-electric modules mounted in series on a water cooled base plate. This electrical device makes use of what is known as the "Peltier" effect, which produces a temperature drop across the elements. According to the manufacturers, the maximum thermo-electric cooling was $-60°C$, but it was found that temperatures as low as $-100°C$ could be obtained when cold brine at $-2°C$ was circulated through the underside of the platen. The copper discs and specimens were kept at $-90°C$ and dried under a vacuum of 10^{-3} torr. Depending on the size of the specimen, the drying would be complete within 3 or 4 hours. The specimen was subsequently removed from the platen and kept in a desiccator.

Miscellaneous techniques. There is considerable interest in this laboratory in the fine structure of pollen grains. The scanning reflection electron microscope is an invaluable tool in such investigations, as it readily reveals the complex surface sculpturing of these structures. However, it is frequently necessary to examine transverse sections of pollen grains to see the construction of the various layers making up the wall.

Studies have shown that it is possible to mix pollen grains with a little water containing a small amount of gum arabic or egg white. The resultant paste may be frozen on a freezing microtome, and a series of sections cut with either steel or glass knives. The sections are thawed, washed free of the gum or egg white, and the cleaned fragments of wall may be easily transferred to the specimen stub and examined in the scanning microscope. The wall sections vary in size and orientation, but it is relatively easy to reconstruct the cross sectional appearance of the pollen-grain wall. The same technique has been applied to fern spores, and there seems no reason why the method should not be applied to other biological tissues. The gum arabic or egg white was found to be necessary to bind the pollen grain-water matrix into a compact mass, and it prevented the frozen mass from falling off the microtome specimen holder (Fig. 21).

Pollen grains have been embedded in wax using conventional methods, and thin sections (2–5 μ) cut on a microtome. Glass or diamond knives are preferable to steel knives as the latter tend to leave scratch marks on the section. The sections are placed on pieces of coverslip and gently dewaxed with xylol. The dewaxed sections were then examined in the scanning microscope. A limited amount of success has been obtained with thin sections of material embedded in epoxy resin. Plant tissue, fixed in glutaraldehyde and osmium and embedded in Araldite, was sectioned at

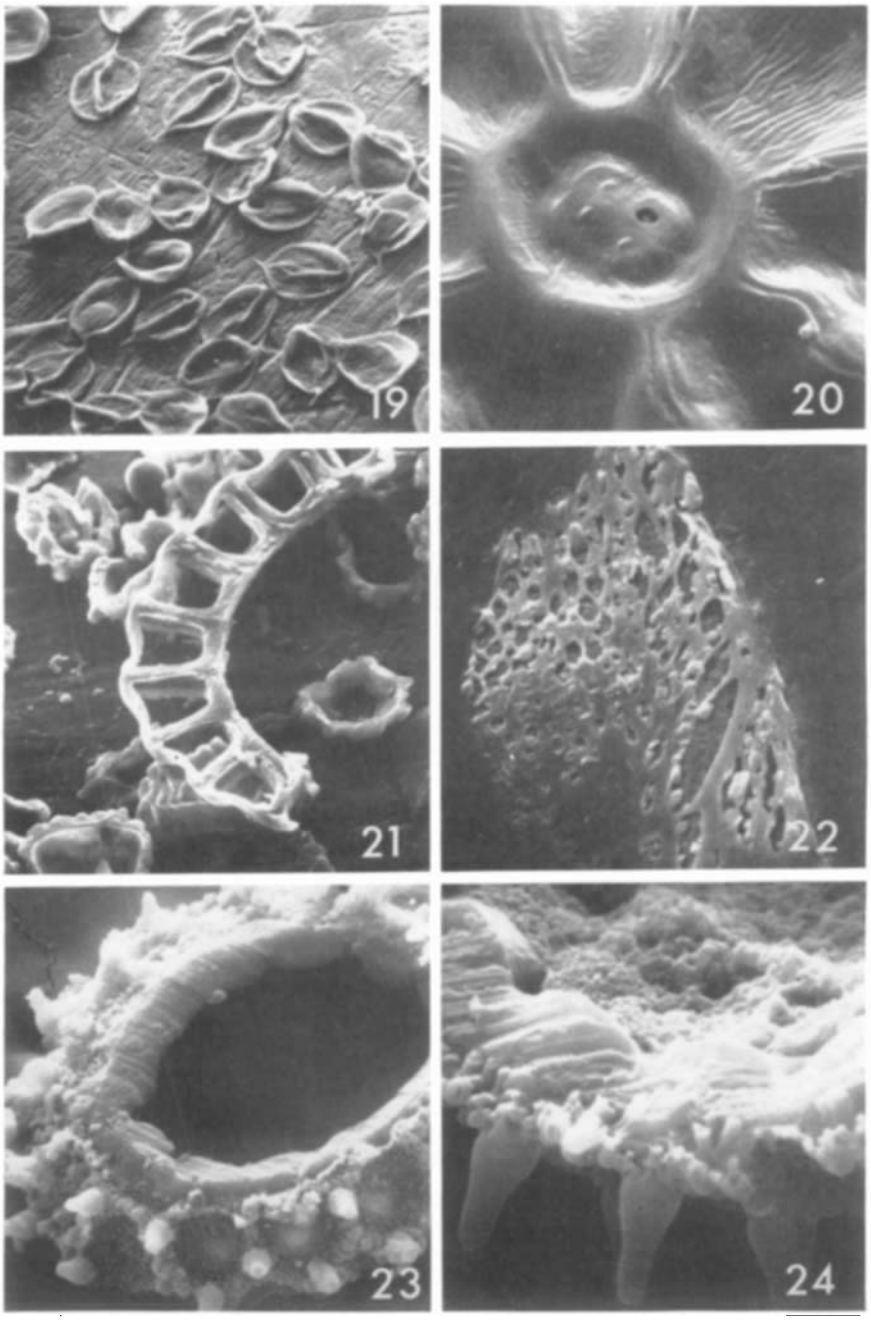

Fig. 19. Collapsed cells of the Euglenoid alga *Phacus* sp. × 220.
Fig. 20. Polystyrene replica of part of the leaf surface of *Limonium vulgare*, × 920.
Fig. 21. Sections of fern spores and sporanium wall, × 4200.
Fig. 22. Resin section of surface of the pollen grain of *Endymion non-scriptus*, × 2400.
Fig. 23. Wax section of *Ipomœa purpurea* pollen, × 760.
Fig. 24. Details of *Ipomœa purpurea* pollen, × 2250.

1000 Å, the sections transferred to pieces of coverslip, and the resin removed using sodium methoxide. The preliminary studies indicate that this method may be applicable to a wide range of plant and animal tissue (Figs. 22–24).

The scanning microscope has been used in the study of leaf surfaces. It has been found extremely difficult to preserve adequately the surface topology of leaf surfaces even by freeze drying methods. The advantage of the technique lies in the fact that large surface areas of leaves may be examined with minimal distortion of their surface relationships. Mr. Brian Chapman in this laboratory has succeeded in making polystyrene surface replicas (Chapman, 1967), which will withstand handling and may readily be coated with carbon or metal films (Fig. 20).

Specimen coating

During examination in the scanning reflection electron microscope, the specimens gradually build up a charge which may distort the image. It is thus necessary to coat the specimens with a film to allow the excess charge to leak away. The film must be a conducting substance of suitable thickness to allow the charge to leak away, but not thick enough to decrease the resolution. It is usual to apply, under a vacuum of 10^{-5} torr, a film, 300–500 Å thick, of a gold-palladium alloy. This method has been found to be quite successful, but is both expensive and time consuming. Sikorski *et al.* (1967) have found a considerable simplication in the preparation of polymer specimens by spraying them with an antistatic aerosol preparation called Duron. This preparation has been found to be quite adequate for instrumental magnifications up to 10,000 diameters, but above this it limits the resolution and the coating film should be the gold-palladium alloy.

Mr. Brian Chapman, working in this laboratory, has achieved some success with thin films of carbon and aluminium. Carbon may be evaporated onto the surface of the specimen at a pressure of 10^{-5} torr followed by a thin coating of aluminium. Alternatively much thicker carbon films may be evaporated at a higher pressure of 10^{-3} torr, in which case it has not usually been found to be necessary to coat with aluminium. These preliminary studies have shown that it is possible to replace the gold-palladium alloy film with a carbon and aluminium film, at least up to instrumental magnifications of 12,000 diameters. For high magnifications and resolution there does not presently appear to be an adequate substitute for the gold-palladium alloy.

It is necessary to rotate the specimen during the brief coating procedure to ensure an even deposition of film. Our experience has shown that the best results were obtained by rotating the mounted specimens at 100 r.p.m. while the angle of the specimen holder was varied during the coating. A simple holder, powered by a fractional horse power motor, may easily be constructed, placed in the evaporating unit and controlled from the outside. Once the specimen has been prepared and suitably coated, it is vital that it should be kept and transported in a dust free environment. It is advisable to blow the surface of the specimen with a small puffer prior to its examination in the microscope.

DISCUSSION

A description has been given of some specimen-preparation methods which have been found to be useful in the examination of plant and microbial material in the scanning reflection electron microscope. There appears to be no reason why these same methods should not be applied to other biological material. The major problem in preparing living material for the instrument is to preserve as far as possible the natural three-dimensional appearance of the specimen. Thus, when

examining a spore or pollen grain it is essential to know that any surface irregularities are inherent in the specimen and not an artifact resulting from faulty preparative techniques. In some instances, such as the detailed examination of part of the surface, this may not be vital, but, in order to put the scanning microscope to its maximum use, the natural shape of the specimen must be preserved. The scanning microscope does not replace conventional replica techniques, which when properly applied will give better resolution by at least one order of magnitude. Replica techniques are a laborious procedure needing great care and, unless high-resolution information is required, a scanning reflection micrograph will give more information in a shorter space of time.

No details have been given of the application of scanning microscopy to specific biological problems. The techniques discussed in this paper have been used in this Department in the examination of unicellular blue-green algæ and bacteria, a wide variety of angiosperm pollen, fossil and living seeds and spores, and living bryophyte and fungal spores.

An extended bibliography is included, and contains a list of papers where the scanning microscope has been used for biological investigations. These papers contain brief details of preparative techniques which may be applicable to other studies.

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