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The Application of Scanning Electron Microscopy to Biological Research

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The application of scanning electron microscopy to biological research

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[Plates 7 to 10]

The various modes of specimen signal acquisition presently available on the scanning electron microscope permit both morphological and analytical studies to be carried out on the specimen. The advantage of the scanning electron microscope is that it provides an information-rich image at resolutions determined by electron optical consideration. This is because of the unique separation of the process of information localization from the information transfer and display. The application of cathodoluminescence and X-ray microanalysis to biological investigations is only now beginning, and the preliminary studies show that these two techniques are going to rapidly provide molecular and elemental information about specimens. Although the ion beam etching process is not completely understood, thus making valid interpretations rather difficult, this process coupled with *in situ* visualization in the microscope will provide a valuable tool in the study of biological material. The comparative ease of specimen preparation for all but the most labile biological material coupled with the ability to examine specimens at low accelerating voltages without a surface coating permits a rapid examination of the surface features of plant and animal material at resolutions of better than 100 nm. More critical preparative procedures are necessary when examining labile biological materials, and details are given of some preliminary studies on the examination of samples at liquid nitrogen temperatures. The simple preparative technique involved coupled with the versatility and controlled sensitivity of the stage modules allows rapid visualization of the specimens.

Introduction

Following the introduction of commercially available scanning electron microscopes in 1965 a large number of papers have appeared which attest to the value of this instrumentation in biological research. A reasonably complete list of specific applications of electron probe instrumentation to biological problems is given by Johnson (1969), Wells (1969, 1970) and in the bibliography compiled by Rossi (1968). The basic instrumentation has been adequately reviewed by other speakers in this meeting, and for further details the reader is referred to the reviews by Oatley (1966) and Thornton (1968). The limitations of space only allow passing reference to the applications of the scanning electron energy analysing microscope to biological studies. Crewe and his co-workers (this volume, p. 61) have elegantly shown that it is possible to determine atomic or molecular species on the basis of the unique energy loss suffered by the electrons as they pass through material. These characteristic energy losses are then used to provide picture contrast. Stroud *et al.* (1969) have applied these techniques to cells and were able to demonstrate that at different levels of energy loss, particular structures are distinguished with high contrast in unstained sections. These and associated techniques will have important applications in the study of cell and molecular structure.

Many of the applications of scanning electron microscopy to biological samples are dependent on adequate preservation of the material before its examination in the microscope column. Some of the newer methods will be described in this present paper, and more detailed preparative techniques are given by Echlin (1968), Boyde & Wood (1969), and Marszalek & Small (1969). As some general reviews on the application of scanning microscopy to biological systems are presently available (Hayes & Pease 1968), this present paper will concentrate on some of the recent advances in tissue preparation and specimen signal acquisition.

Topographical detail

The bulk of the information available from scanning electron microscopy falls within this category (see figures 1 and 2, plate 7). The interaction of the high-voltage primary electron beam with the specimen gives rise to low-voltage secondary electron emission which may be collected and the information displayed on a cathode-ray tube. As far as topographical detail is concerned, the images are closely similar to those formed by reflected light optical microscopy, but with a much greater depth of focus and at considerably increased resolution. The secondary electrons which form this signal have low energies of the order of 4 to 6 eV. Some of the primary electrons may penetrate the specimen, and following scattering through large angles, may re-emerge from the surface as back-scattered electrons. Such electrons have undergone little energy loss and may in turn generate further low-energy secondaries which may lessen the overall quality of the picture.

However, the primary excited secondary electrons form a large proportion of the total signal, and since they are derived from an area only marginally wider than the irradiating electron probe, fine detail is obtained from the surface of specimens. The resolution is considerably improved if the specimen is coated with some suitable conducting material such as evaporated carbon or a gold/palladium alloy. At the accelerating voltages normally used in the examination of biological material most of the secondary electrons are derived from within a few tens of nanometres of the surface of the specimen. The amount and nature of the secondary electrons depends on a number of factors including the angle of the incident beam, the atomic number of the specimen, and the surface properties of the specimen. Some degree of topographic information may also be obtained utilizing the primary electrons which are back-scattered from the specimen. Such electrons show little change in energy from the primary beam. The characteristics of the back-scattered primaries are dependent on the nature of the specimen. The lower the atomic number of the specimen so the greater the absorbance of the primary electrons. On a flat surface this provides a measure of element discrimination as elements of low atomic number appear lighter in contrast than those of high atomic number.

Cathodoluminescence

On removal of the scintillator from the electron collector system or by adding an additional photomultiplier and light guide, it is possible to detect luminescence emitted naturally from the specimen or as a result of excitation by the primary beam. The light can arise either from the natural luminescence of the material or from artificially introduced phosphors. Unfortunately the efficiency of production and collection of photons is low and the generation of light quanta takes place in a volume considerably bigger than indicated by the cross-sectional parameters of the primary beam. This generation of light quanta is lowered even further if thin sections are used, and it is necessary to have long exposure times in order to build up an acceptable picture image. Many of the dyes which have been used so successfully in fluorescent light microscopy are readily quenched when used in a cathodoluminescence system which adds to the difficulties of obtaining a picture. Cathodoluminescence closely resembles fluorescence optical microscopy and although very little of the former has been carried out on biological systems, it would appear that the same parameters and characteristics may be applied to both systems. Thus low concentrations of excitable material are needed to provide visible light which

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means that specific flurochromes may be added to living cells in quantities too small to cause toxic damage. Although the system has a high contrast the resolution is only marginally better than 100 nm and it is difficult to see how any accurate *quantitation* may be achieved.

In the absence of any definitive studies it is difficult to predict all the applications of cathodoluminescence. On the intrumentation side we presently have a system with a resolving power of between 80 and 100 nm and a depth of focus which is better than that for reflected light optical microscopy at comparable magnifications by a factor of at least 300. Provided a number of specific resistant dyes are found the potential uses of cathodoluminescence would appear to fall into two main divisions. First, the observation of naturally occurring luminescent structural material in plant and animal tissues and the localization of any luminescent by-products of their metabolism. Secondly, where fluorescent stains which have a specificity for a particular region of the cell or for a particular molecular species are added to living or fixed cells. These secondary luminescent studies may take the form of staining well-defined cellular entities or end products of reactions in tracer techniques or for detecting foreign substances and used in conjunction with labelled antibody methods.

X-ray microanalysis

Significant advances have been made in the X-ray microanalysis of some of the low atomic elements such as Na, K, Mg, Ca, which are commonly found in biological material. X-rays emitted from the specimen as a result of excitation by the electron probe are analysed to determine their chemical composition. The spectrum of the X-rays produced is characteristic of the elements in the target. Thus by monitoring the spectra of the X-rays produced as the specimen is scanned by the electron beam it is possible to build up a picture of the elemental distribution in the specimen (figures 3 and 4, plate 7). Suitable crystals in spectrometers are used for the dispersive analysis of the longer wavelength X-rays, and there are now suitable solid-state proportional counters for measuring the energy of X-rays with a resolution approaching 100 eV. The advantage in biological studies of the non-dispersive detectors is that it is possible to use much lower beam currents to detect elements, thus avoiding the possibility of engendering thermal and radiation damage within the specimen.

One limitation to the X-ray mode of operation is that since the X-ray production is a bulk effect the resolution is limited to about 500 nm, which is well within the range of optical light microscopy. Attempts to improve the resolution by using a finer electron probe or thinner specimens have not met with much success because of the lower rate of X-ray production. Nevertheless, the X-ray microanalysis mode of operation does provide a sensitive device for detecting elements. It is difficult to give meaningful figures to illustrate the sensitivity of the system since detection and measurement depend on the other elements which can give a confusing background signal. Hall & Höhling (1969) give the limits of detectability in terms of both minimal absolute amounts (10^{-15}–10^{-16} g) and minimal weight fractions (10^{-3}–10^{-4}, i.e. 0.1–0.01%) within a volume of 1.0 μm^3. The appropriate instrumentation is available which permits quantitative analysis of single points on a specimen surface, semiquantitative analysis of single line scans across the specimen, and for qualitative distribution of selected elements over a scanned raster. The selected elemental distribution may be displayed on a second video display unit, and can be compared directly with the signal obtained from secondary electron emission which provides topographical information concerning the specimen. The detection and localization of

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artificially introduced specific heavy metal stains may be a useful adjunct to X-ray microanalysis. The specificity of silver salts for halides, the stochiometric binding of uranium to DNA, the affinity of lead salts for RNA containing nucleoproteins, and the availability of ferritin-coupled antibodies, to name but a few, show that this aspect of the technique may provide yet another tool for microanalytical studies.

Ancillary techniques

In the past few months a number of ancillary techniques and smaller pieces of instrumentation have become available for use with the scanning electron microscope which are of considerable use to the biologists. Three of these devices and techniques will be described, and although they will be dealt with separately, it will become clear that they are most profitably used together.

Low voltage operation

Boyde & Wood (1969) emphasize that the scanning microscope should not operate at as high an accelerating voltage as possible when examining biological material. A complex interplay of factors determine the operating conditions and, more importantly, the optimum amount of information which may be obtained from the specimen. By operating the primary beam at high accelerating voltages this permits a reduction in probe diameter with a consequent increase in resolution. However, this increase in beam voltage also leads to deeper penetration by the beam, which in turn may on a highly ornamented sample give rise to secondary electron emission from a depth of several micrometres instead of 20 to 30 nm. These secondaries will contribute spurious brightness variations in the image which can give rise to errors in interpretation. Another consequence of deeper penetration is that it may give rise to uneven specimen charging which will either emit more secondaries or will deflect the primary beam. The net effect of this uneven charging is uneven contrast and problems of astigmatism at higher magnifications. The contrast variations, like the brightness variations, make meaningful interpretations more difficult.

However, these are extreme examples for it is usual to apply a conductive coating of carbon or some heavy metal to the specimen before its examination in the microscope. The conductive coating of metal serves a number of functions, all of which improve the specimen image. The metal layer allows any electrical or thermal build up to leak away and is in itself a better emitter of secondaries than the underlying lower atomic number of the biological material. But the application of the metal coat, although improving the information in the emissive mode, precludes obtaining meaningful data using the cathodoluminescent and X-ray microanalysis mode of operation.

Boyde & Wood (1969) maintain that for examining biological material the accelerating voltage should not exceed 10 kV. At this voltage the resolution is in the 20 to 30 nm range, but this is accompanied by a dramatic decrease in artefacts. Much has been talked and subsequently written in the present symposium about improving the *resolution* of electron optical image, and only passing reference has been made about the *information content* of the specimen. One of the outstanding advantages of scanning electron image formation is that it provides a much wider range of information transfer systems than is available from transmission electron optical systems, albeit at a reduced resolution.

In some instances, as will be seen later, it is not practicable to apply a surface coating, and

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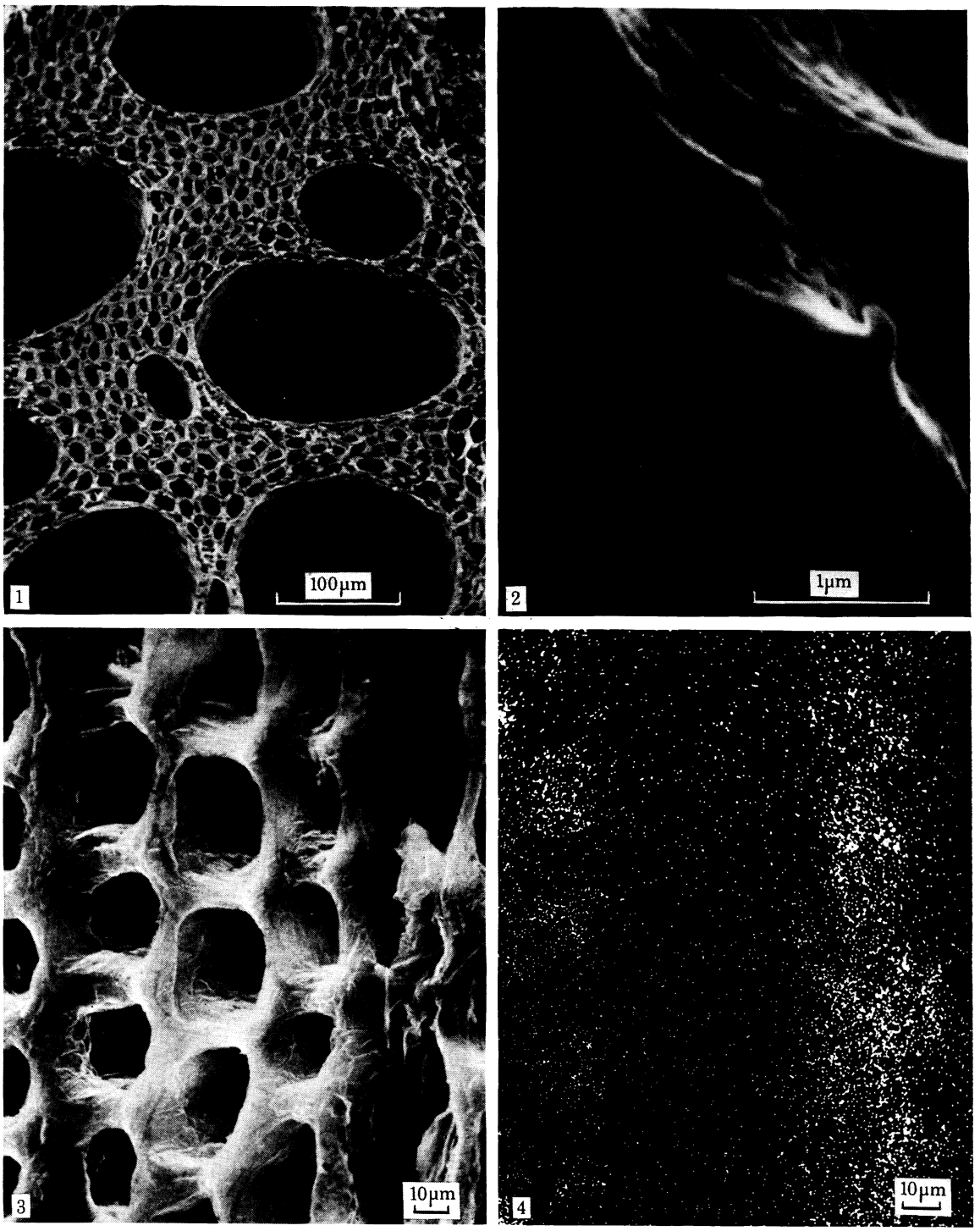

FIGURE 1. Transverse section of *Vitis vinifera* stem. Carbon coated. Emissive mode. 20 kV.

FIGURE 2. Detail of *Ambrosia elatior* pollen grain. Pd/Au coated. Emissive mode. 10 kV.

FIGURE 3. Transverse section of wood impregnated with cuprinol. Carbon coated. Emissive mode. 10 kV.

FIGURE 4. Transverse section of wood impregnated with cuprinol. Carbon coated. X-ray microanalysis for Cu. Kα note high concentration of copper in ray parenchyma cells.

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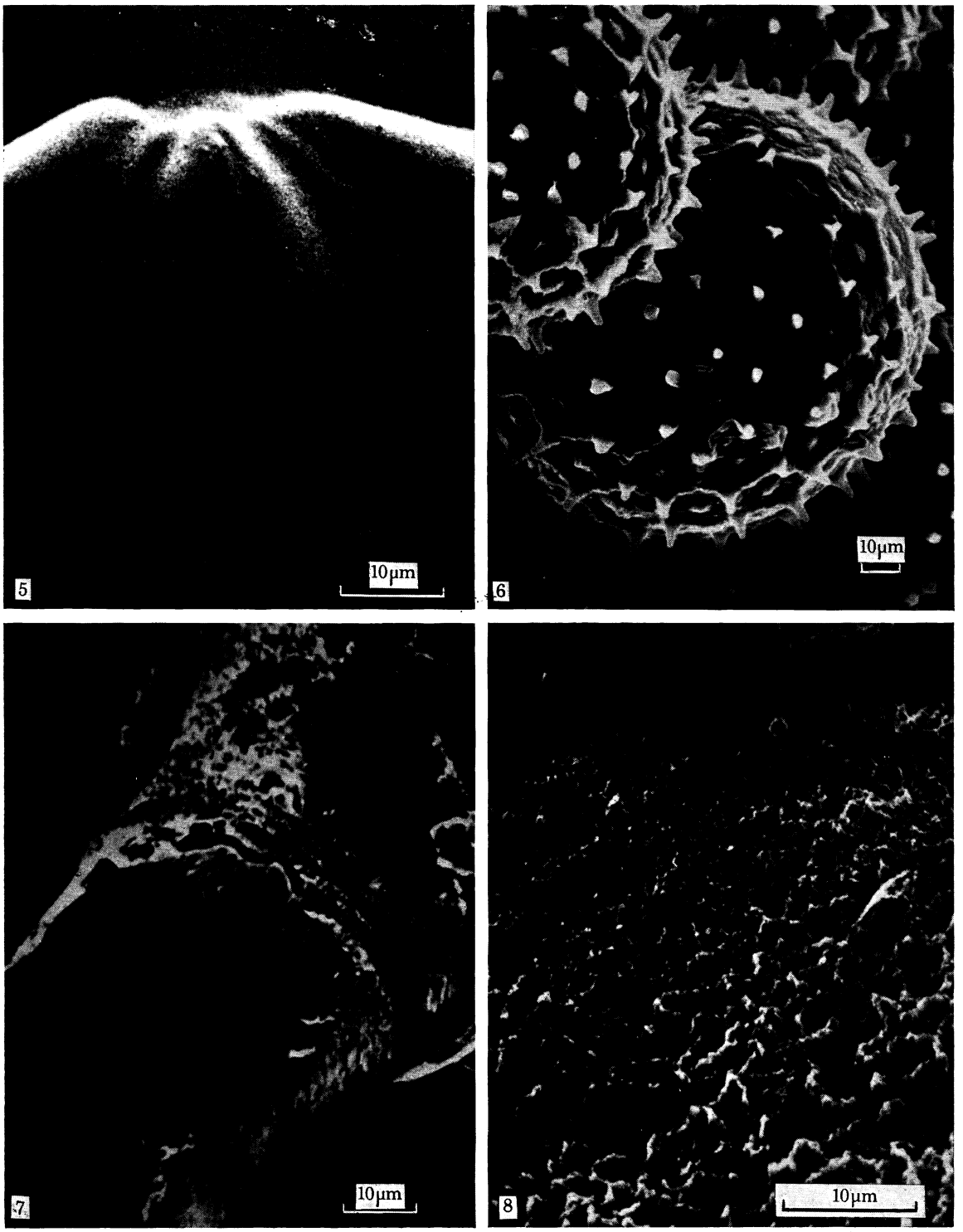

FIGURE 5. Stomatal pore of *Tradescantia bracteata*. Uncoated. Emissive mode. 1.0 kV.

FIGURE 6. Pollen grain of *Ipomoea purpurea*. Uncoated. Emissive mode. 2.0 kV.

FIGURE 7. Ion beam etched greenfly leg at junction of coxa and trochanter. Uncoated. Emissive mode. 10 kV .

FIGURE 8. Ion beam etched *Quercus ilex* leaf. Uncoated. Emissive mode. 10 kV.

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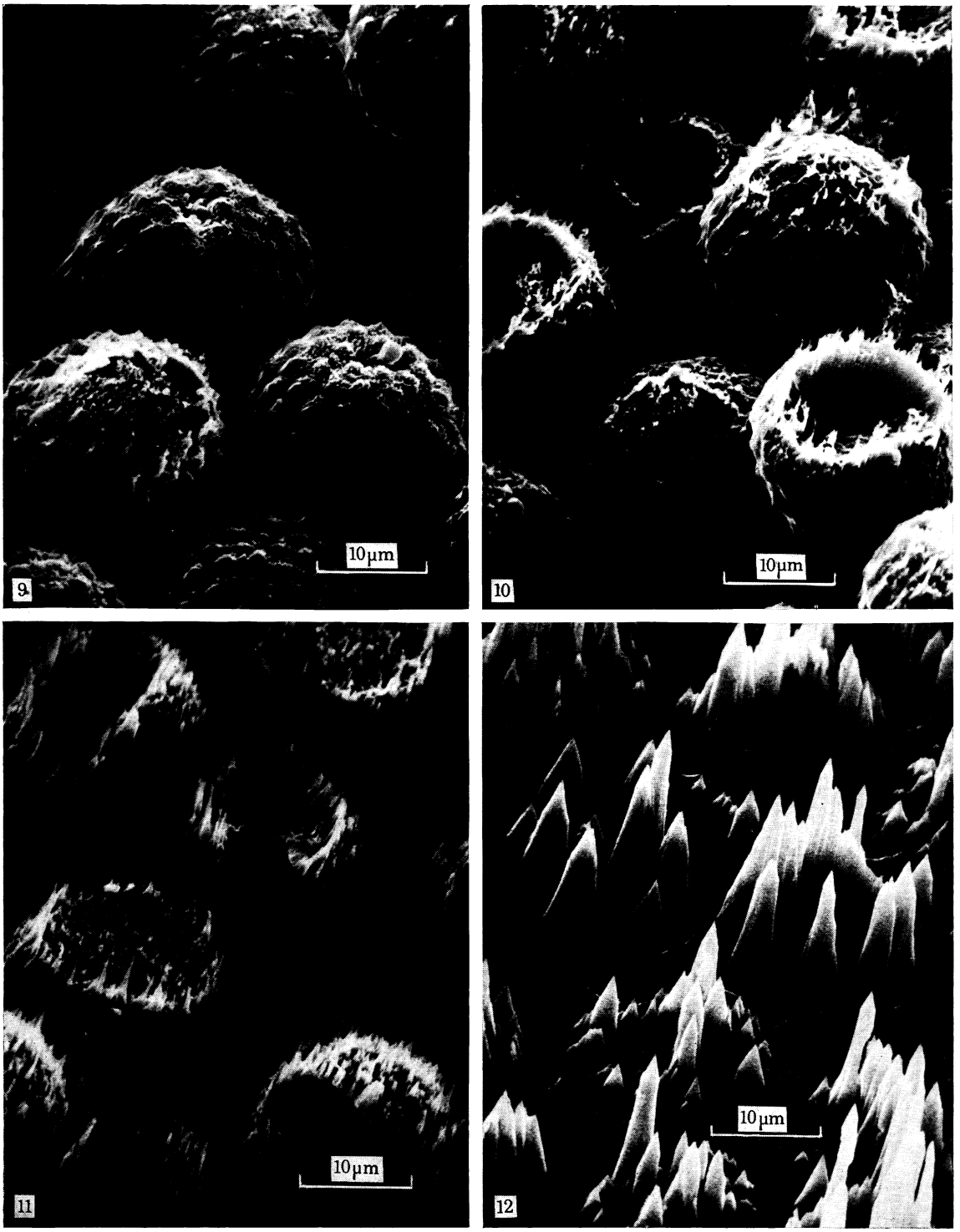

FIGURES 9 to 12. Progressive ion beam etching of *Ambrosia elatior* pollen grains. Emissive mode. Pd/Au coated. 20 kV.

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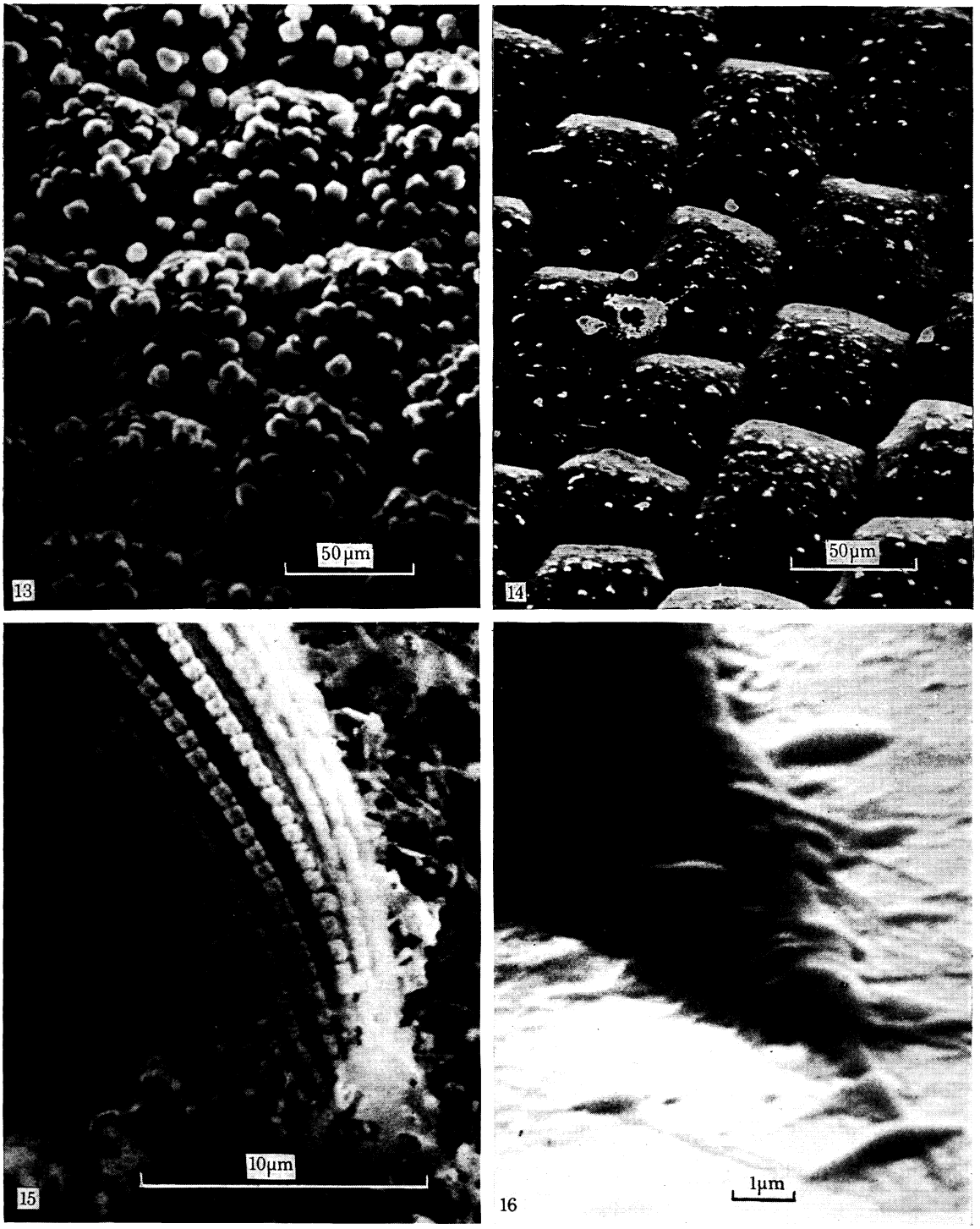

FIGURE 13. *Lagarosiphon major* leaf covered with ice. Uncoated. Emissive mode. 10 kV. Temperature = −175 °C.
FIGURE 14. *Lagarosiphon major* leaf free of ice. Uncoated. Emissive mode. 10 kV. Temperature = −95 °C.
FIGURE 15. *Euglena spirogrya*. Uncoated. Emissive mode. 10 kV. Temperature = −120 °C.
FIGURE 16. Mouse L. tissue culture cells. Uncoated. Emissive mode. 10 kV. Temperature = −95 °C.

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in order to avoid the adverse charging effects, it is necessary to operate at considerably reduced accelerating voltages. We have carried out a series of experiments in which fresh material was examined uncoated at an accelerating voltage of between 1 and 2 kV at a resolution of between 80 and 90 nm (see figures 5 and 6, plate 8). There is considerably less beam penetration and, more importantly on biological material, a considerable reduction in the radiation flux impinging on the specimen.

As well as examining fairly hard biological material, such as pollen grains and insect cuticle, it has also been possible to view hydrated materials such as mesophyll leaves. Heslop-Harrison & Heslop-Harrison (1969) were able to observe considerable detail in an untreated leaf of the xerophyte, *Dianthus plumarius*, and attributed some of their success to the ability of the vacuum system in the Stereoscan scanning microscope to take up any water which is evaporating from the specimen. With mesophytes and hydrophytes, although there was considerable cellular collapse due to the loss of water, the presence of dissolved minerals and ions in the cell sap are thought to have contributed to the generation of secondary electrons, and provided a conductive pathway. The advantage of the low voltage operation is that it allows a rapid examination of material at medium resolution without the disadvantage of the time-consuming preparative and coating techniques. Even fairly labile specimens may be examined provided this is done within a few minutes of their being introduced into the microscope column.

Ion beam etching

It is now possible to selectively remove parts of specimens inside the microscope column. An ion source has been designed to operate in the specimen chamber of the Stereoscan scanning electron microscope (Echlin, Kynaston & Knights 1969). The ion source is a demountable cold cathode argon discharge unit which may be focused onto any area within the dimensions of the specimen stub. Using this ion source the internal morphology of a number of complex resistant biological objects has been examined (figures 7 and 8, plate 8). An examination of ion beam etched *Ambrosia* pollen grains (figures 9–12, plate 9) show that the first signs of etching are in the small openings of the tectum. As the etching proceeds, these holes are progressively enlarged and within a short time the tectum is entirely removed. The underlying bacula are revealed not as solid pillars, but as short spikes, and it is thought that some etching of these structures takes place before the tectum has finally disappeared. Once the exinous pollen grain wall has been removed by the ion beam, the cellulosic intine is rapidly disintegrated, revealing the underlying cytoplasm which is even more quickly removed.

The advantage of having the ion beam source within the microscope is that it is possible to carry out a controlled etching process and immediately examine the uncoated specimen at low accelerating voltages. Should high resolution results be required, the specimen may be removed from the column, given a metal coat, and examined. Further etching may then be carried out as it is a simple process to remove the metal coating.

There are a few problems which still remain, particularly regarding the correct interpretation of the image. It is not entirely clear whether the ion beam removes material by a charring and sputtering process, or whether the mechanism is one of true vaporization. When the ion beam hits the specimen it may cause it to accumulate a positive charge. This results in a deflexion of further ions and a consequent diminished etching in this region, whereas other non-charged regions will continue to etch. One way to prevent this would be alternately to

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pulse the ion beam and the electron beam to give a neutralizing effect, but this would also give rise to considerable contamination of the scintillator. It is also clear that two quite different etching profiles may be obtained if the etching is carried out at two different angles. These differences are thought to be related to the crystallinity and substructure of the specimen. Crystalline materials have differential rates of etching along different crystal planes.

Finally, it is obvious that soft tissue etches much more rapidly than hard tissue. It is possible to have the situation where a small piece of resistant material protects an underlying layer of soft tissue, which everywhere else is being rapidly eroded away. This gives rise to the 'mushroom effect' with a small piece of material perched on top of a long column.

In order to obtain meaningful interpretation of ion etched material, it is necessary to distinguish between the differences due to selective etching based on tissue resistance, the difference arising from variations in the crystallinity and substructure of apparently homogeneous materials, and the uneven charging effects of the ion beam.

Examination of specimens at low temperatures

In close collaboration with physicists from Cambridge Scientific Instruments some preliminary studies have been made on a versatile temperature controlled stage module which has been developed for the Stereoscan scanning electron microscope. The preliminary design aspects of the stage are given in the paper by Echlin, Paden, Dronzek & Wayte (1970).

A wide range of labile biological tissue has been examined in the microscope at temperatures ranging from +25 °C to −180 °C. The two principal methods of specimen preparation were as follows. Initially it was found that biological material could be quench-frozen by plunging specimen stubs with the material mounted on the surface into liquid nitrogen at −196 °C. With plant material such as leaves of *Lagarosiphon major* (an aquatic angiosperm), and *Tradescantia bracteata* (a terrestrial angiosperm) and root hairs of *Sinapis alba* and *Zea mays*, adequate preservation was obtained with little or no evidence of ice-crystal damage. It is clear that the cellulose wall of plant cells is sufficiently robust to prevent or retard extensive ice-crystal formation and growth.

These procedures were unsatisfactory for optimal preservation of micro-algae, protozoa and animal tissue and cells. Such extremely hydrated and delicate tissues were drained or damp-dried and either placed directly onto cooled aluminium specimen stubs or onto clean platinum disks which in turn were fixed to cooled specimen stubs by a spot of silver-dag. The specimen stubs were then rapidly plunged into liquid Freon 22 (monochlorodifluoromethane) maintained at its melting-point of −140 ° C by liquid nitrogen. The Freon 22 quench freezing invariably gave specimens free of ice-crystal damage. A mixture of isopentane and methylcyclohexane although an adequate quench coolant, was unfortunately transferred via the specimen stub into the microscope and it was impossible to maintain an adequate vacuum in the instrument.

It has been found to be undesirable to infiltrate specimens with cryprotective substances such as dimethyl sulphoxide, polyvinylpyrrolidone and glycerol which have been shown to substantially lower the nucleation rate of water. Although these chemicals are incorporated into material before their preparatory cryofracture techniques, their low vapour pressure precludes their presence in specimens which are going to be introduced into the microscope column operated at room temperatures because they are a source of contamination and may obscure specimen detail. However, although their mobility will be considerably reduced at low

temperatures, we have been unable to demonstrate any advantage to using them before the quench freezing. Heat is only extracted from samples through the surface and as a consequence one only obtains vitrification within the upper 1–2 μm. Below this depth there is always the danger of ice-crystal damage.

Moor (this volume, p. 121) has shown that there are alternative ways to reduce the critical freezing rate other than by using cryoprotective substances. Such methods include ultra-rapid quench freezing at a rate of at least 10^4 K s^{-1}, and the application of high pressure during the freezing process which lowers the rate of ice-crystal growth. It is now possible to obtain water vitrification to a depth of about 200 μm from the surface of a specimen, and as the emissive mode of scanning microscopy only obtains meaningful information from within a few tens of nanometres of the surface, the low-temperature preparative techniques are more than adequate for the available instrumentation technology.

Following the quench freezing the specimen stubs were quickly (within 4–5 s) transferred to the cold stage maintained at -180 °C with liquid nitrogen and the microscope column pumped down to 13 mN m^{-2} (10^{-4} Torr) its working vacuum. An immediate examination of the specimens revealed that they were covered with ice (figure 13, plate 10). This water is derived from two sources. In the case of aquatic organisms or cells maintained in an aqueous medium, the water was part of their immediate environment. Such organisms were either examined in their natural state, or carefully washed in isotonic salts solution to remove any surface debris. A certain amount of atmospheric water will condense onto the specimen during the transfer from the quench-coolant to the specimen stage. We were initially concerned at the presence of this water, and elaborate steps were taken to construct a dry environment specimen transfer chamber. But as will be shown it is relatively easy to remove the surface water within the microscope so these cumbersome procedures were abandoned. The temperature of the stage was raised to between -100 and -90 °C whereupon the high vacuum within the microscope column caused the surface water to sublime revealing the tissue surface below (figure 14, plate 10). The removal of water had to be carefully monitored, and the temperature was not allowed to rise above -85 °C. If too much water was removed, by allowing too great a rise in temperature, then the tissue collapsed. The same tissue showed considerable collapse and surface artefacts when fixed in buffered glutaraldehyde and then taken through a freeze-substitution procedure. Examination of material on the cold stage dramatically demonstrates that water is a vital structural component in many plant and animal cells.

The high stability of the specimen stage and the fine temperature control mechanism allowed the removal of ice to be accurately monitored either by direct observation or by following the temporary increase in pressure in the specimen chamber. Surprisingly large amounts of water could be removed from the specimen and in no instance did we observe or experience inoperatively high pressures. When the removal of the surface water was completed, and this usually took place within 3 to 5 min of placing the specimen on the cold stage, the temperature of the stage was maintained at between -140 and -100 °C during the examination of the specimen.

All the specimens were examined in the emissive mode of operation over a wide range of accelerating voltages without the benefit of surface coating. Attempts were made to spray 'Duron' antistatic spray onto the surface of the *Tradescantia* leaves before quench-freezing. This procedure was abandoned as it gave such consistently poor results because the microdroplets of the antistatic agent obscured any surface detail. In spite of the absence of a surface

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coating it has been possible to obtain a resolution of the order of 100 nm. Although accelerating voltages as high as 30 kV were employed, it was usual to take pictures at 20 kV or lower as this reduces the energy impinging on the specimen. It was possible to measure the radiation dosage per unit area falling on the specimen—a feature of particular importance when examining biological material.

Under typical operating conditions the total power dissipated in the specimen within the volume occupied in the electron range is of the order of a microwatt. For biological materials this can correspond to the power density of the order of 1 W per gram dry mass, or 1 J/s^{-1} per gram dry mass.

The cold stage has been particularly useful in examining micro-algae and protozoa, because they retained their three-dimensional shape while in the frozen state. It was possible to see details and the orientation of the pellicular warts on the myonemes of *Euglena* (figure 15, plate 10) and the array of cilia on *Paramecium*. Preparation of these objects by more conventional means invariably resulted in serious collapse. The cold stage has been particularly useful in examining tissue culture cells. Mouse L. cells, which are sensitive to adverse conditions, were grown on platinum disks, washed several times with a buffered isotonic salts solutions, before being quench-frozen. The cells retain their natural configuration, and show such features as the nuclear bulge (figure 16, plate 10) and the delicate protoplasmic protuberances which are believed to be associated with the attachment of the cells to the substrate. It is also possible to see the fine filamentary appendages at the tips of the cells. Examination of the sample at different tilt angles relative to the primary electron beam revealed further information about the specimen. These preliminary studies clearly demonstrate the usefulness of the cold stage in the examination of biological material and the instrumentation and procedures employed ensure that the specimens faithfully retain their natural three-dimensional configuration. The only treatment which has been applied to the specimens is a quench-freezing technique which has already proven satisfactory in the preservation of cells and tissues. The results so far obtained are often better than results obtained by fixation and freeze-drying techniques. An advantage of the latter technique is that specimens may be coated before examination, thus improving both resolution and ease of interpretation. A distinct advantage of the cold stage is that images may be obtained within a few minutes of the specimen being removed from its natural environment.

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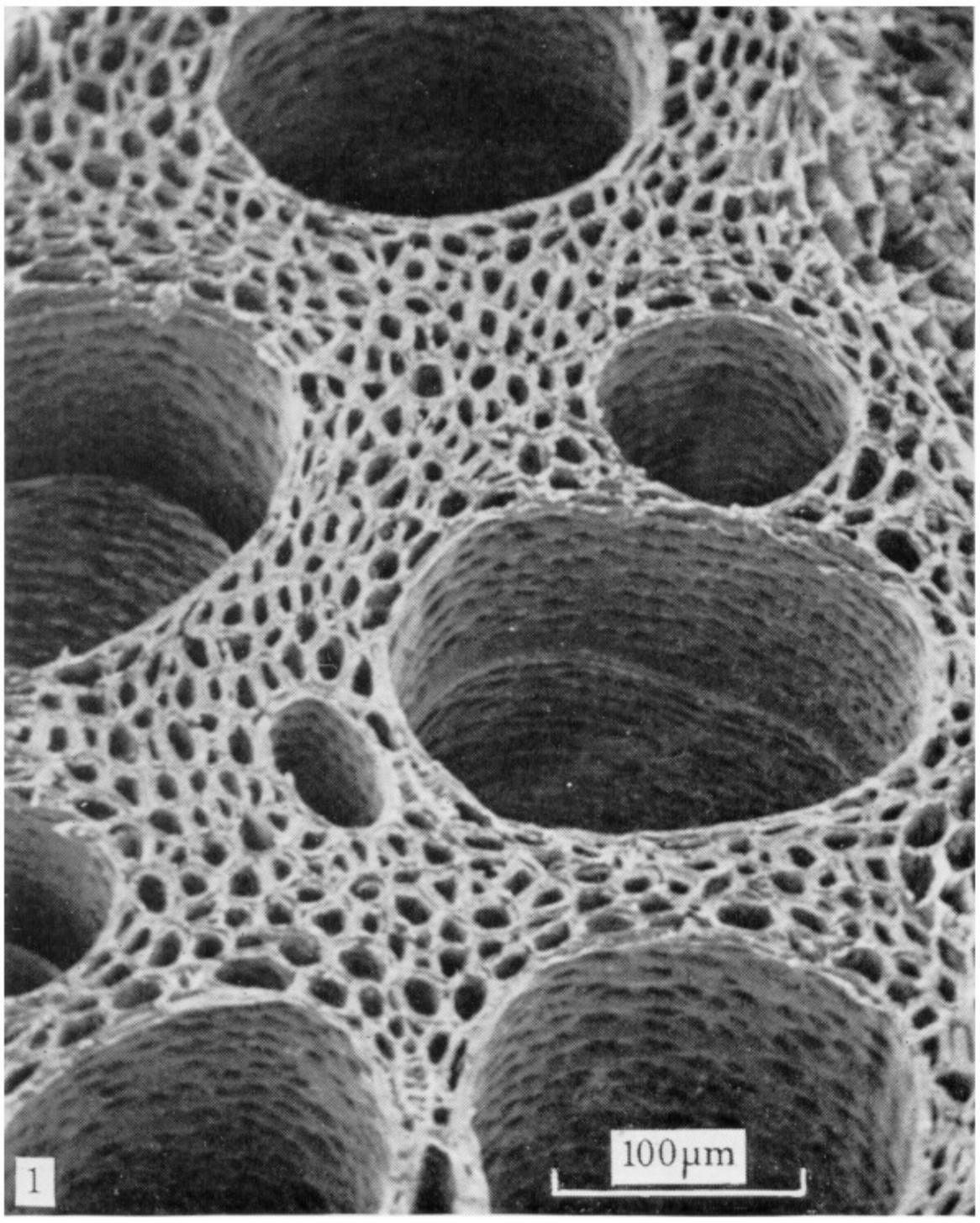
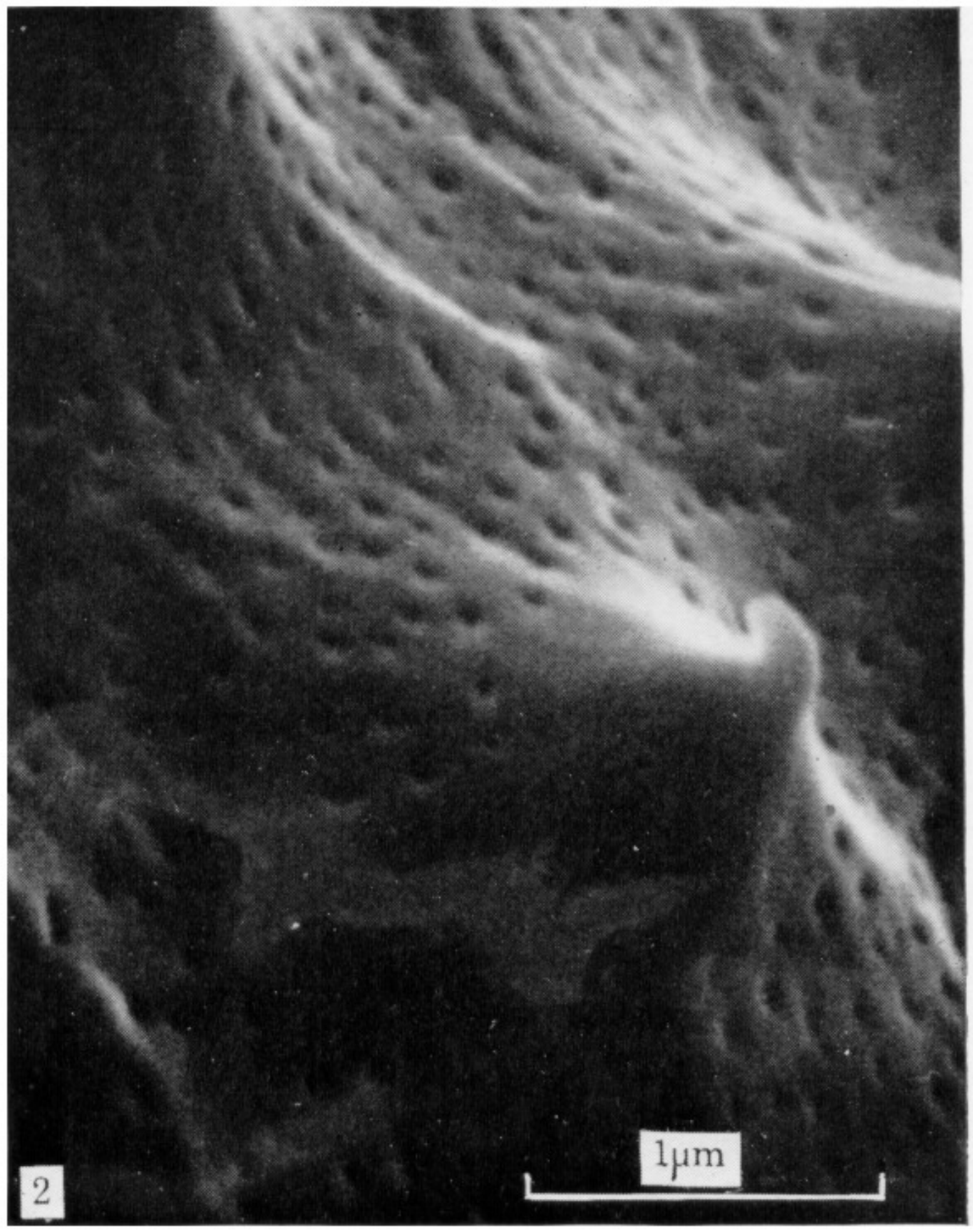
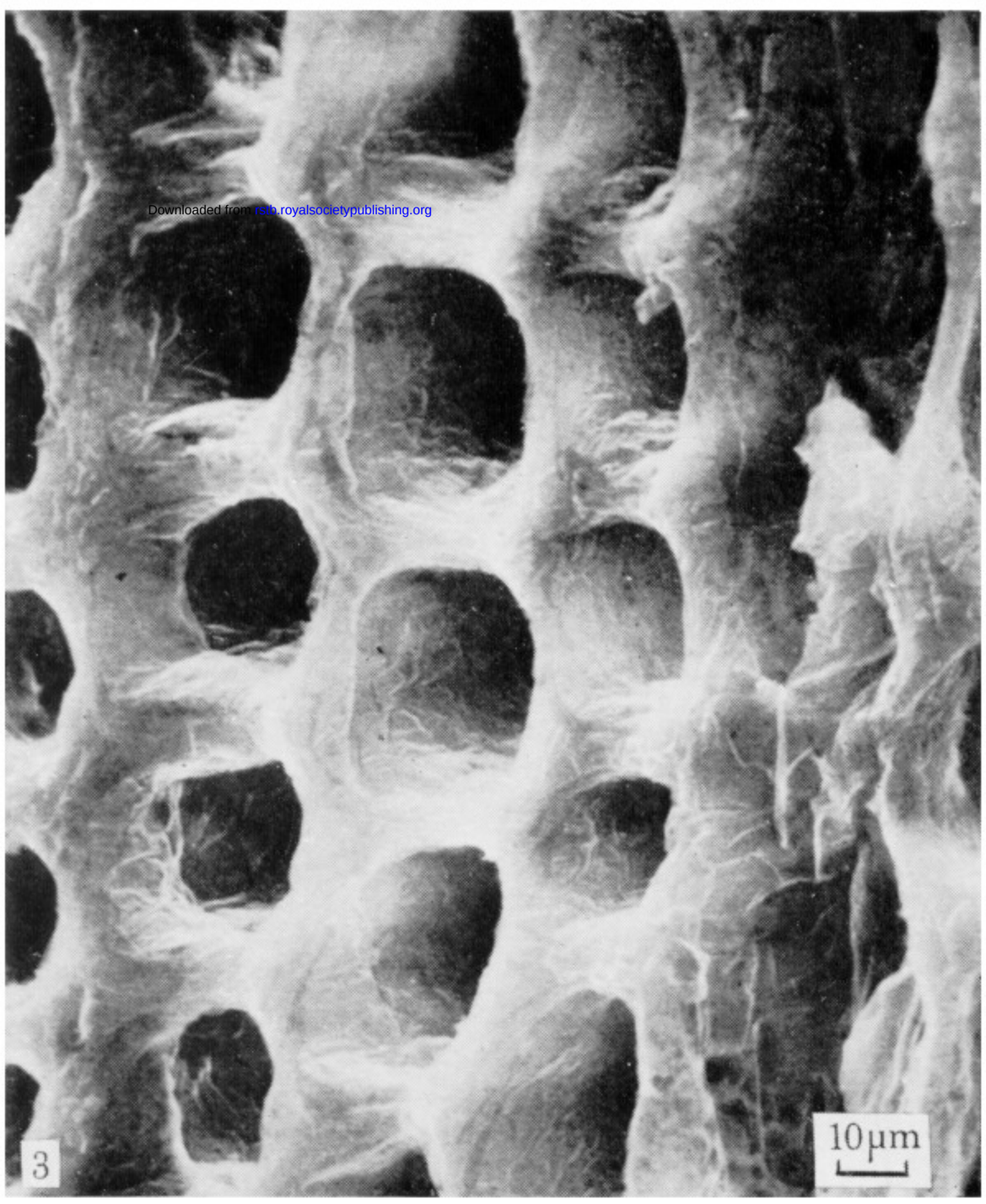






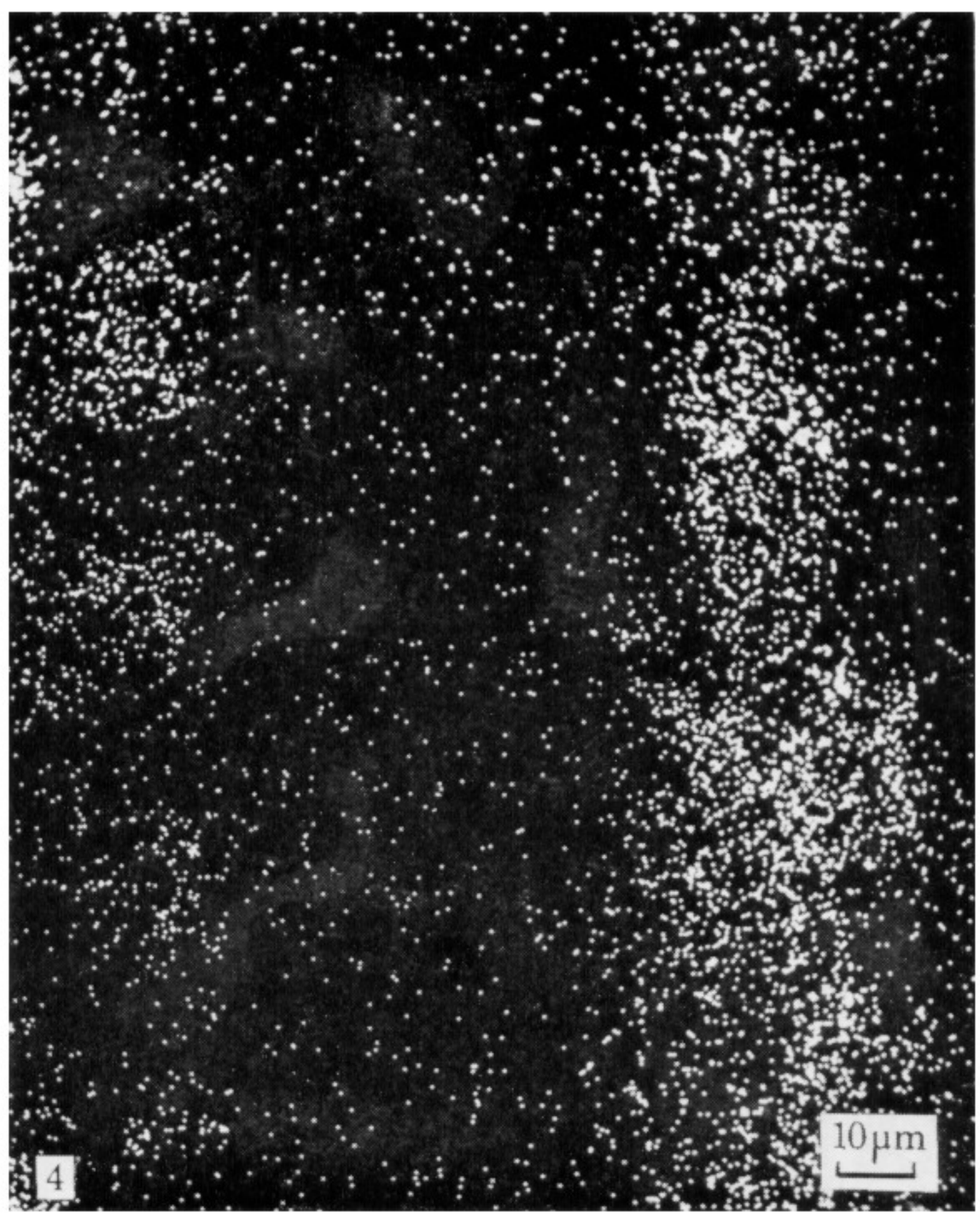
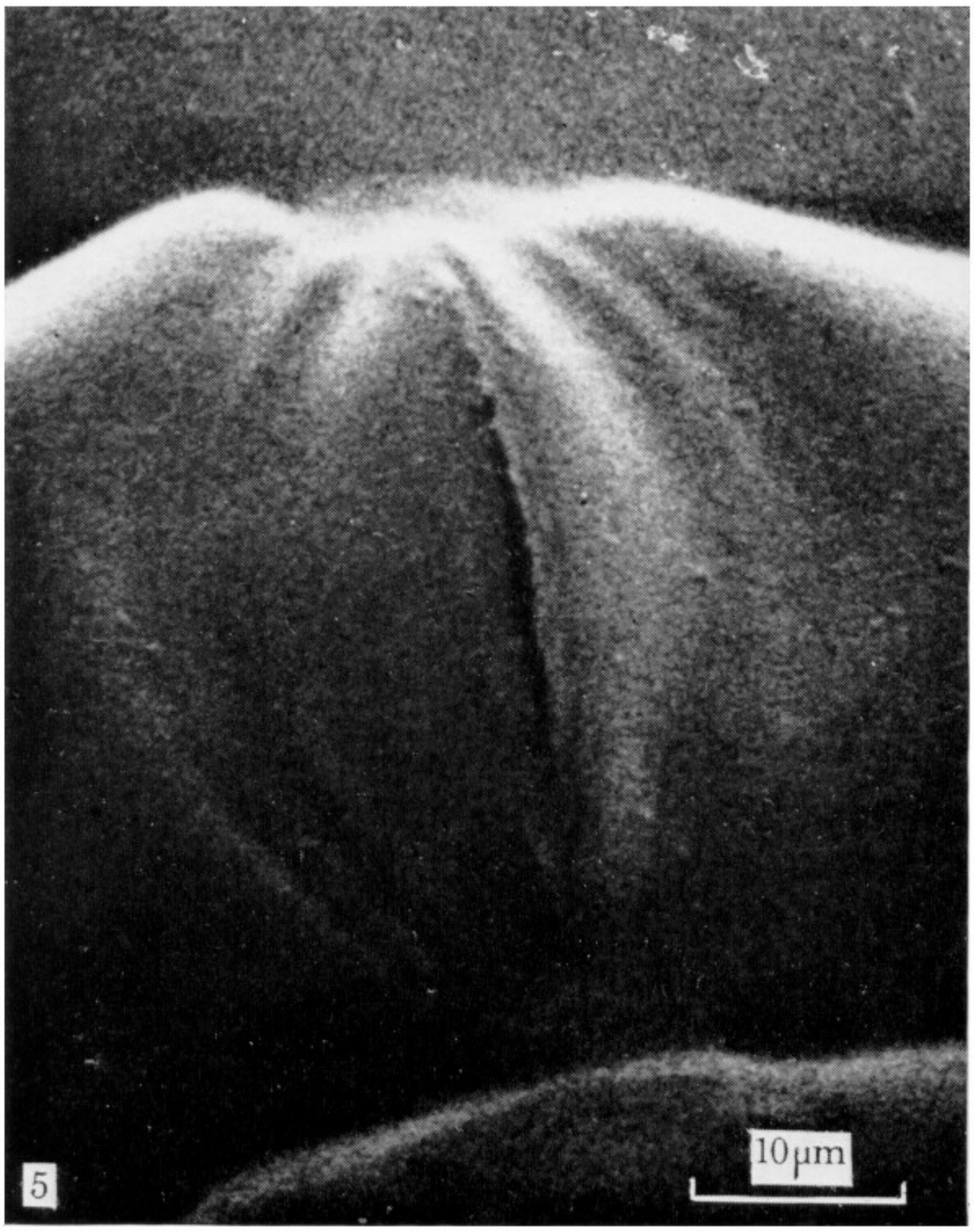


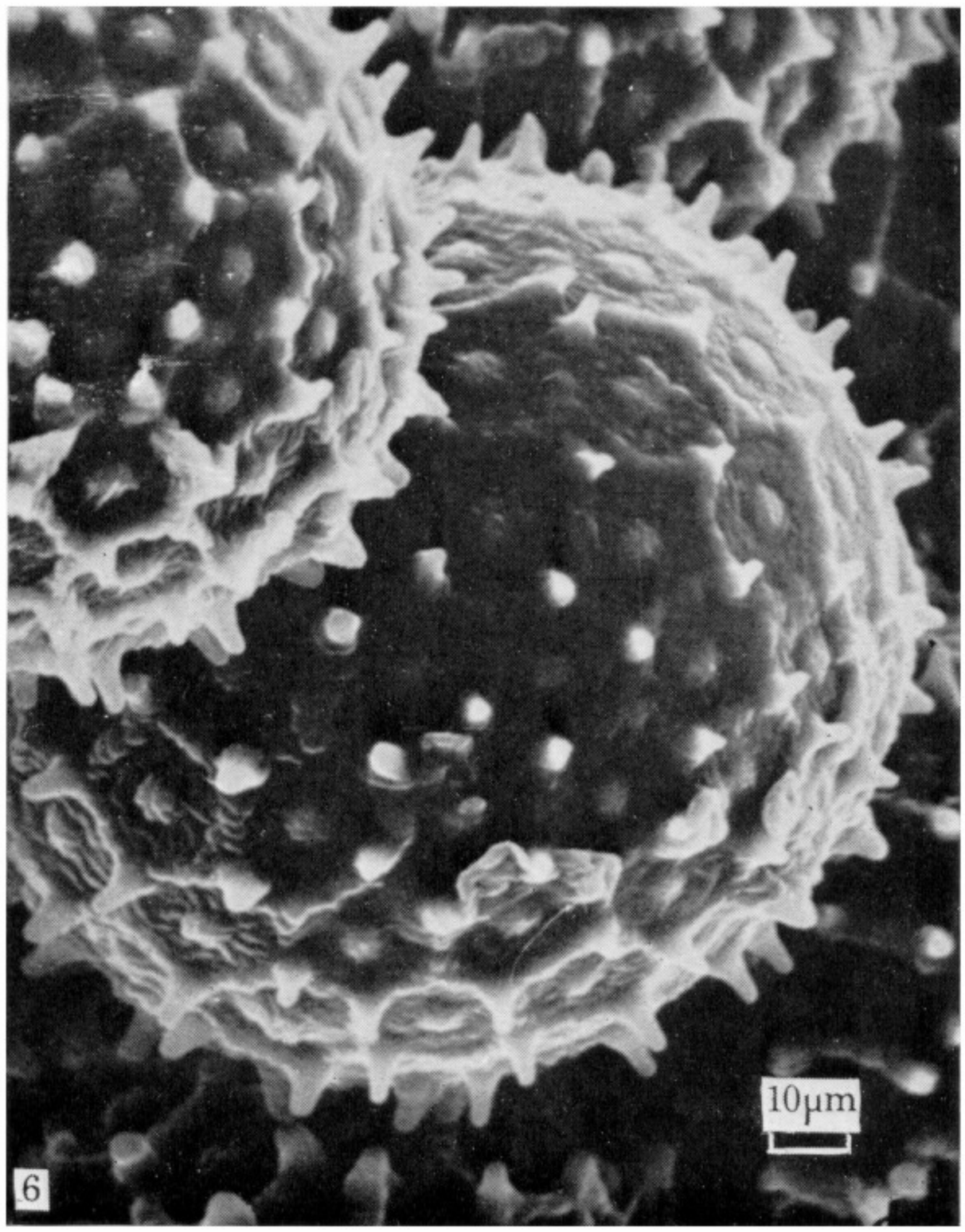
FIGURE 1. Transverse section of *Vitis vinifera* stem. Carbon coated. Emissive mode. 20 kV.

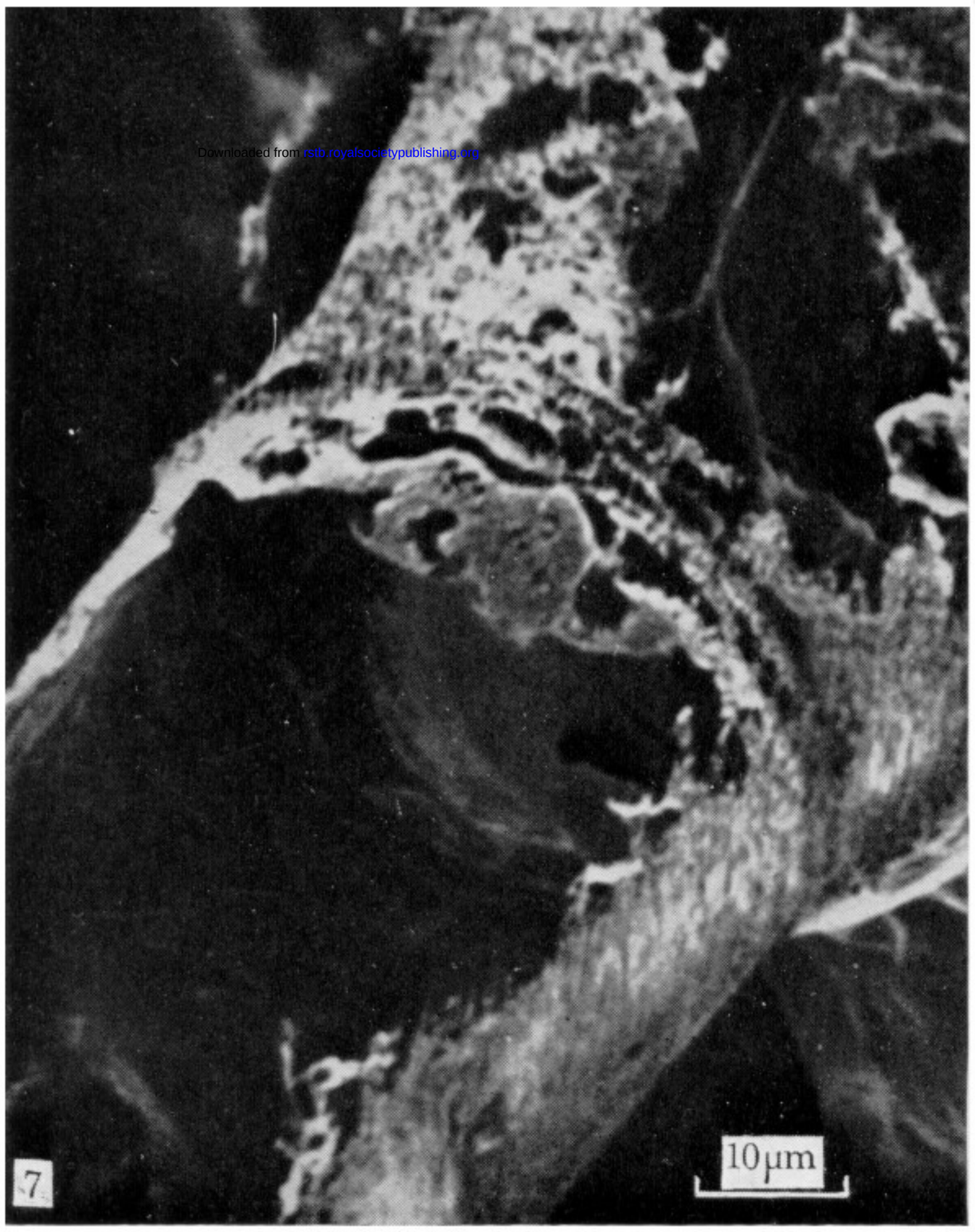
FIGURE 2. Detail of *Ambrosia elatior* pollen grain. Pd/Au coated. Emissive mode. 10 kV.

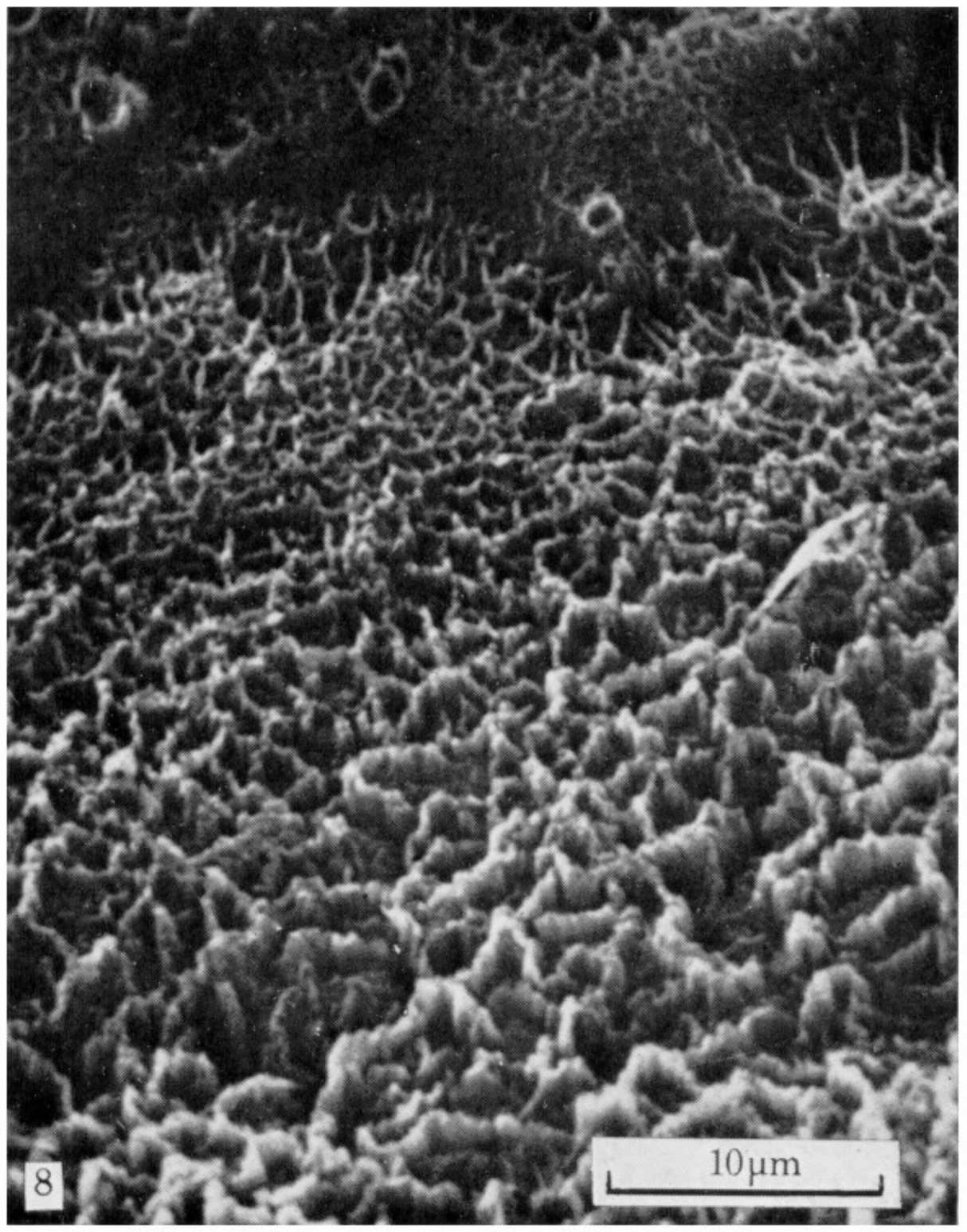
FIGURE 3. Transverse section of wood impregnated with cuprinol. Carbon coated. Emissive mode. 10 kV.

FIGURE 4. Transverse section of wood impregnated with cuprinol. Carbon coated. X-ray microanalysis for Cu. Kα note high concentration of copper in ray parenchyma cells.









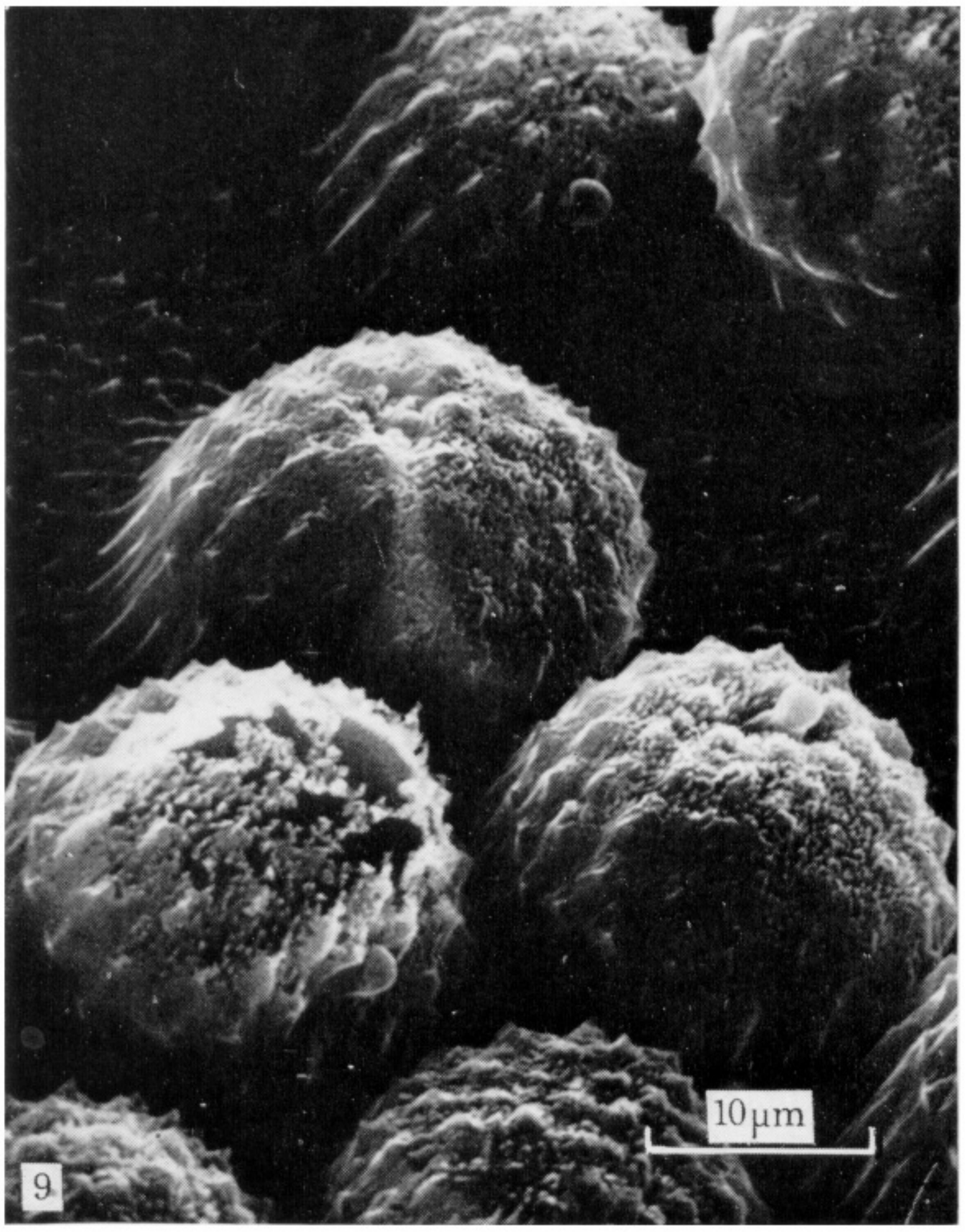
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

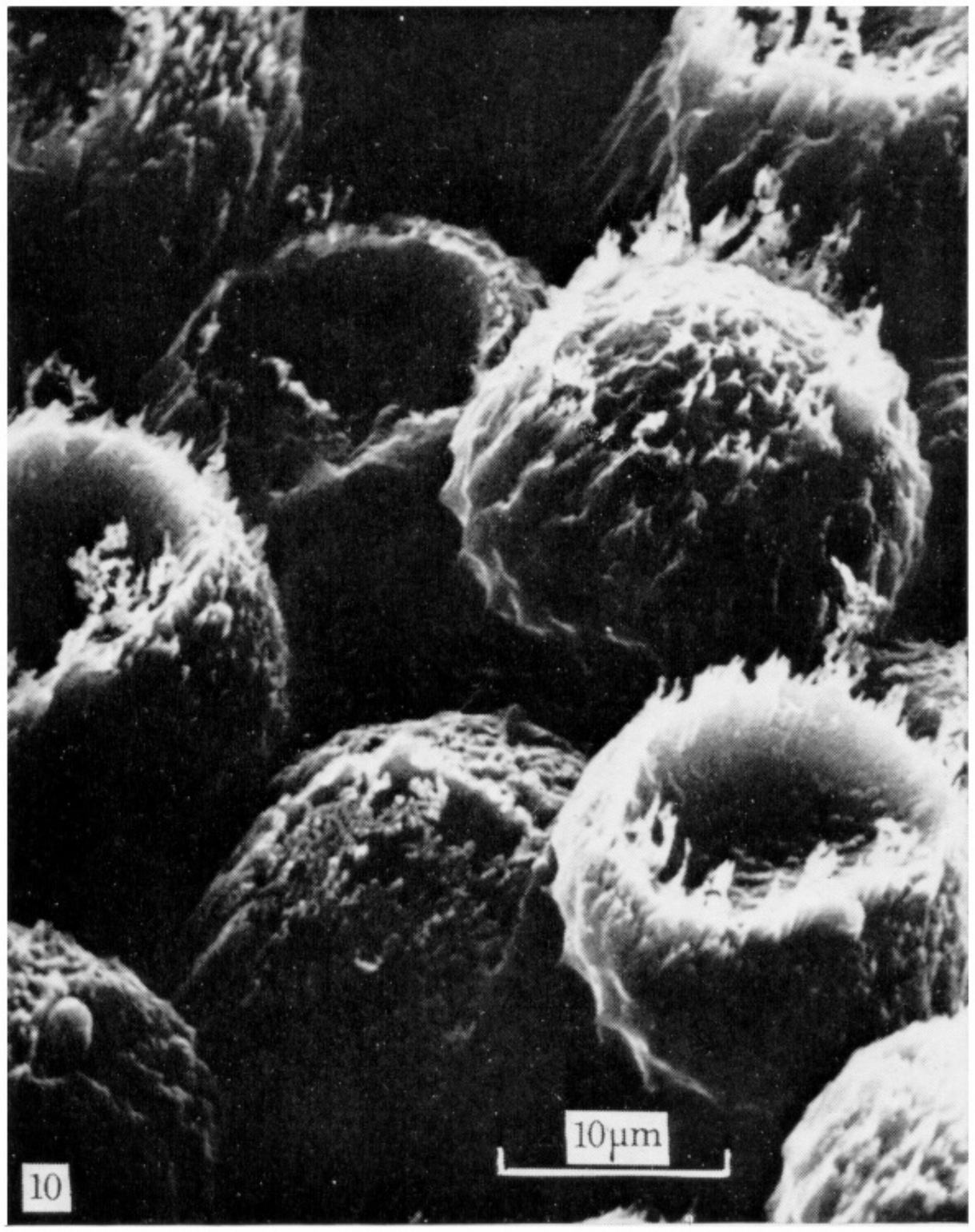
FIGURE 5. Stomatal pore of *Tradescantia bracteata*. Uncoated. Emissive mode. 1.0 kV.

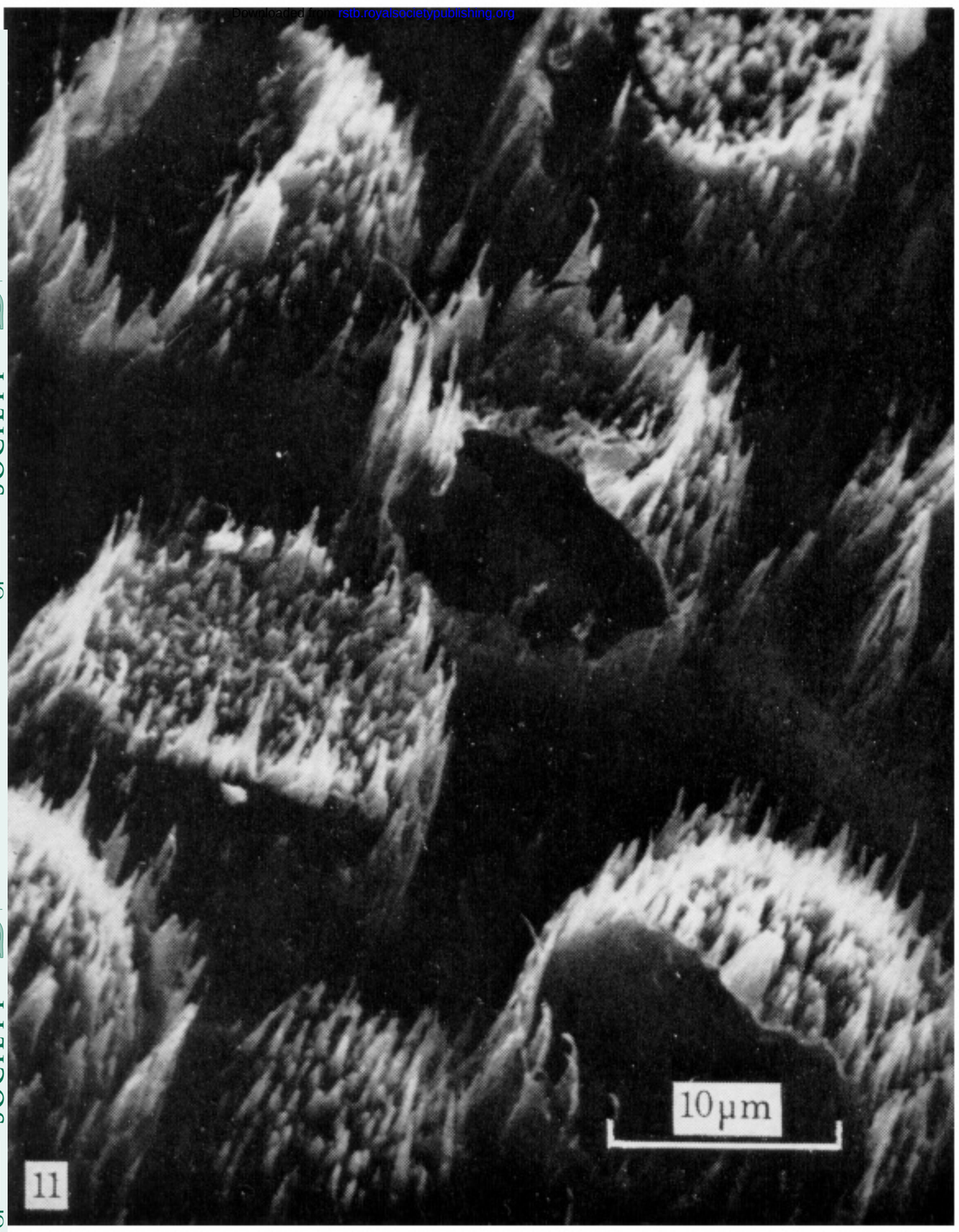
FIGURE 6. Pollen grain of *Ipomoea purpurea*. Uncoated. Emissive mode. 2.0 kV.

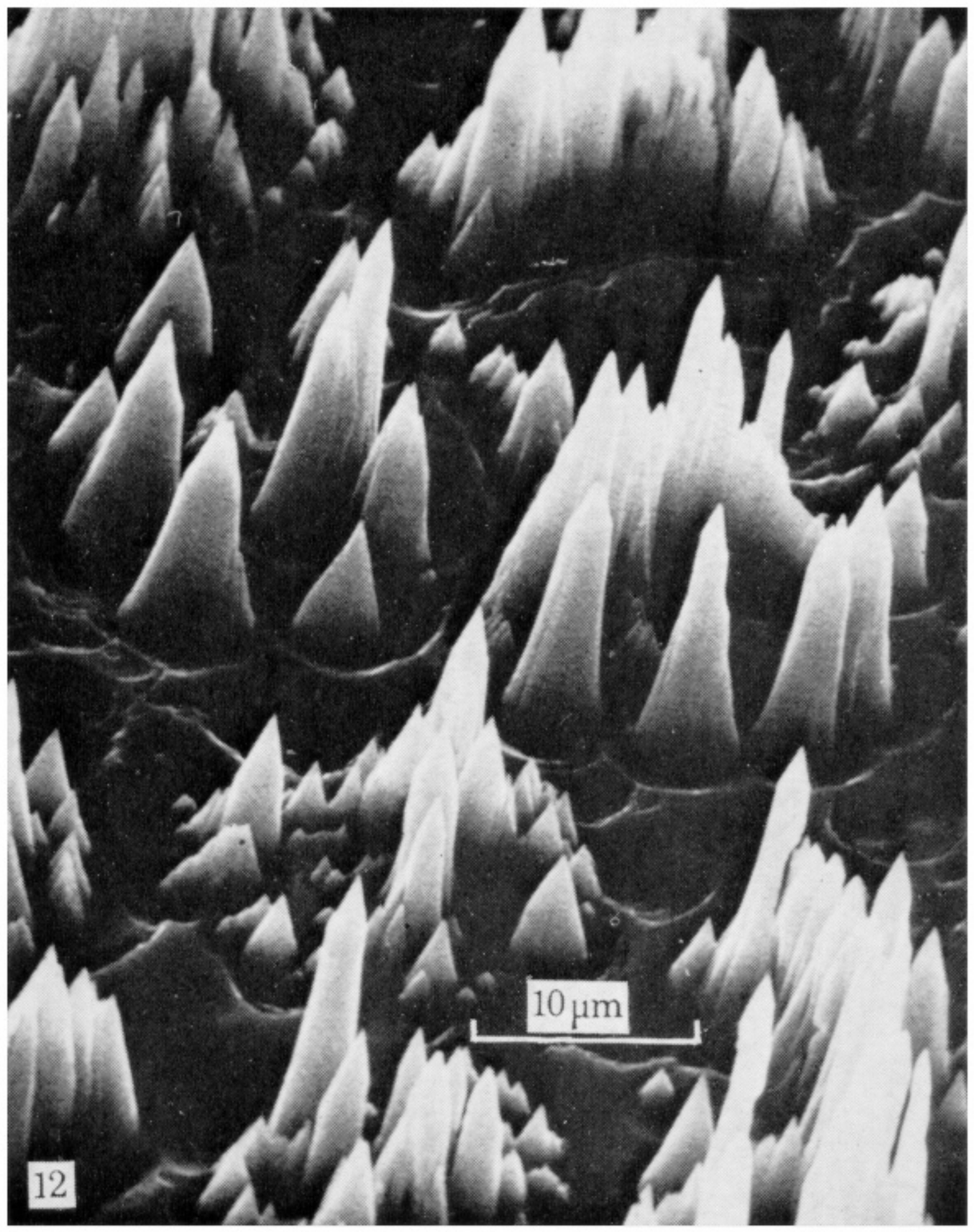
FIGURE 7. Ion beam etched greenfly leg at junction of coxa and trochanter. Uncoated. Emissive mode. 10 kV .

FIGURE 8. Ion beam etched *Quercus ilex* leaf. Uncoated. Emissive mode. 10 kV.

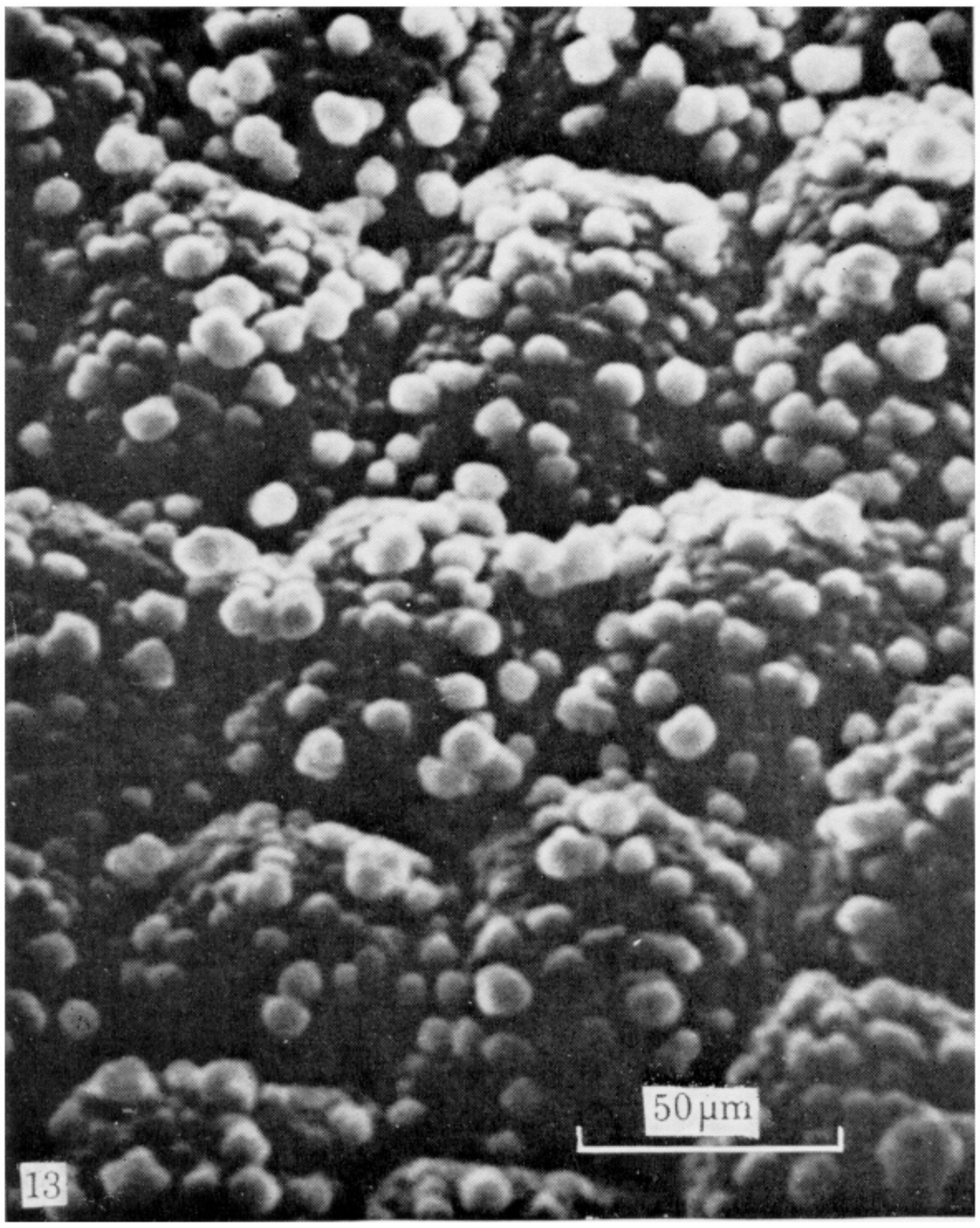








FIGURES 9 to 12. Progressive ion beam etching of *Ambrosia elatior* pollen grains. Emissive mode. Pd/Au coated. 20 kV.



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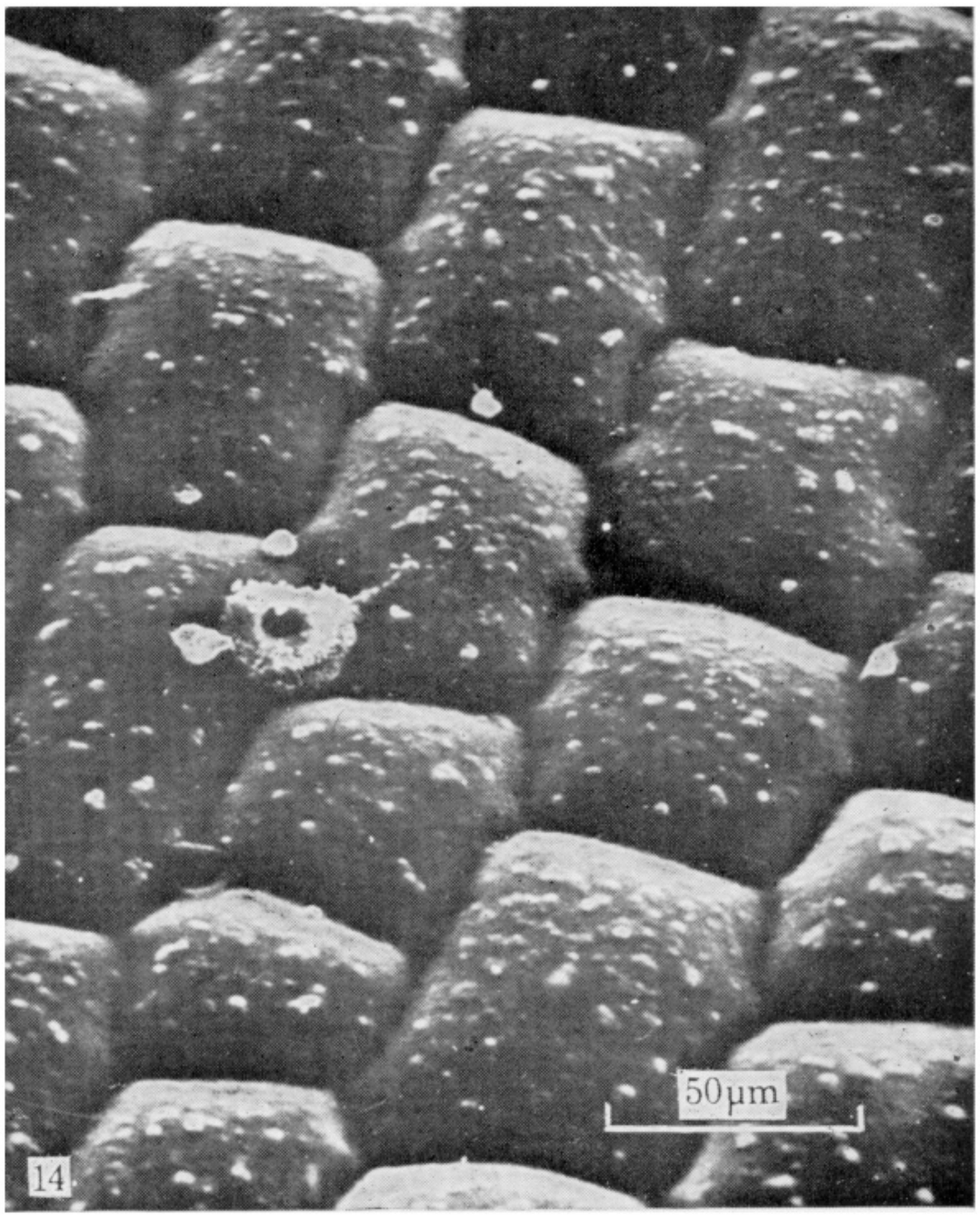

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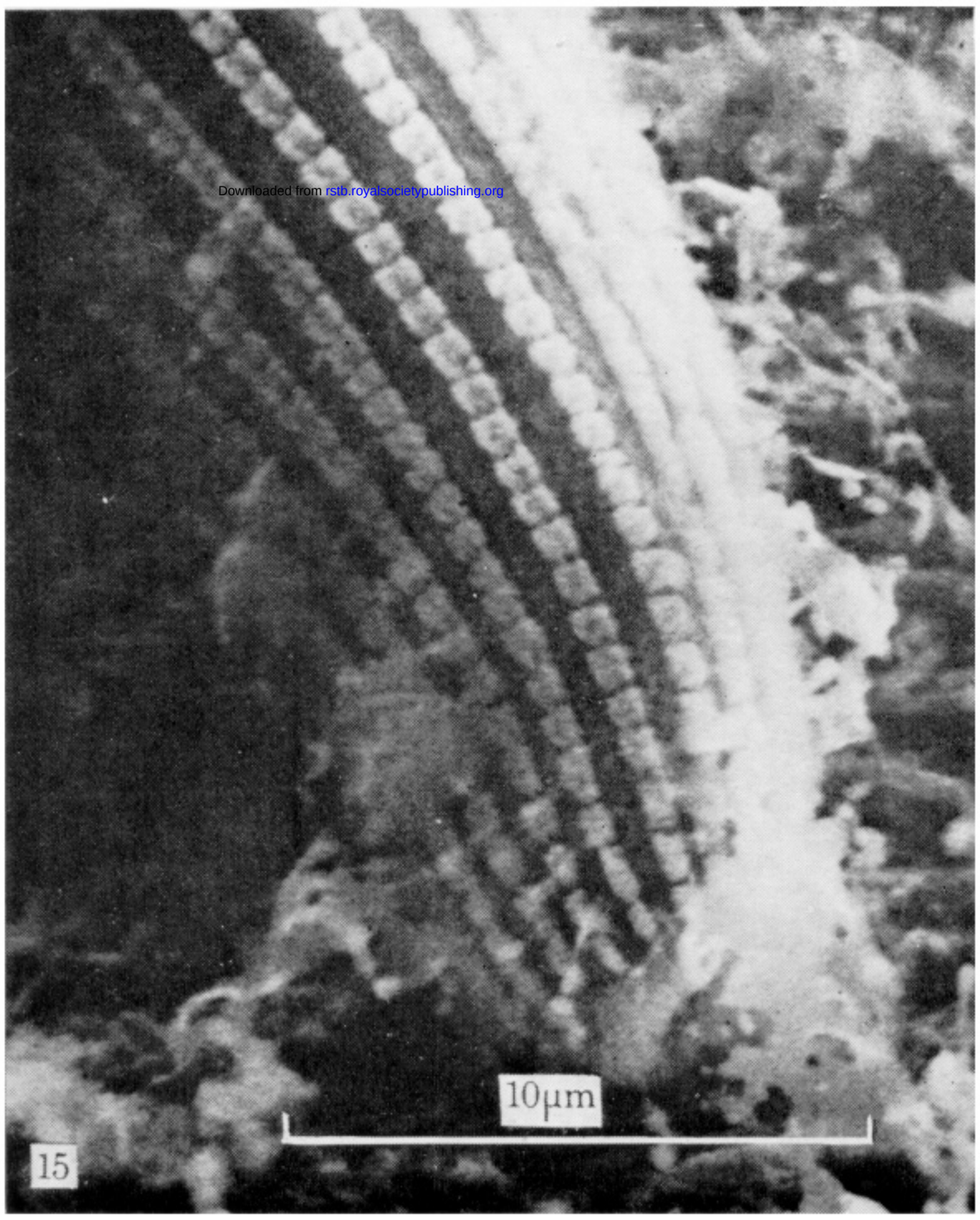

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FIGURE 13. *Lagarosiphon major* leaf covered with ice. Uncoated. Emissive mode. 10 kV. Temperature = −175 °C.

FIGURE 14. *Lagarosiphon major* leaf free of ice. Uncoated. Emissive mode. 10 kV. Temperature = −95 °C.

FIGURE 15. *Euglena spirogrya*. Uncoated. Emissive mode. 10 kV. Temperature = −120 °C.

FIGURE 16. Mouse L. tissue culture cells. Uncoated. Emissive mode. 10 kV. Temperature = −95 °C.