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The interpretation of scanning electron micrographs

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The problem of the interpretation of images of biological objects in the scanning electron microscope is discussed. The influence of preparative techniques, drying and coating methods on the final image is illustrated by reference to higher plant protoplasts. Methods for confirming the presence of new structural detail are suggested. An attempt is made to illustrate and introduce the need for a higher standard of interpretative and critical skill in the presentation of results obtained by means of scanning electron microscopy.

INTRODUCTION

The scanning electron microscope (SEM) was initially used in biology for the study of gross morphological features of cells and organisms, such as the surface structure of leaves, pollen grains and whole cells. Many of the results obtained from such work presented little interpretative difficulty since they were often merely a novel view of features already known or suspected from light microscopy or transmission electron microscopy (TEM). Scanning instruments now available have significantly improved performance in terms of resolution, and this development offers many opportunities for examining new problems in cell biology. From the simple description of whole cells, research has moved to the demonstration of T4 bacteriophage on the surface of *E. coli* (Amako *et al.*, 1974), the release of influenza virus at high resolution (Amako, 1975) and the structure of polytene chromosomes (Brady *et al.*, 1977). Structural observations now embrace surface details of bacterial cells (Amako and Umeda, 1977) and SEM is also used to analyse the results of experimental treatments (Overton, 1977).

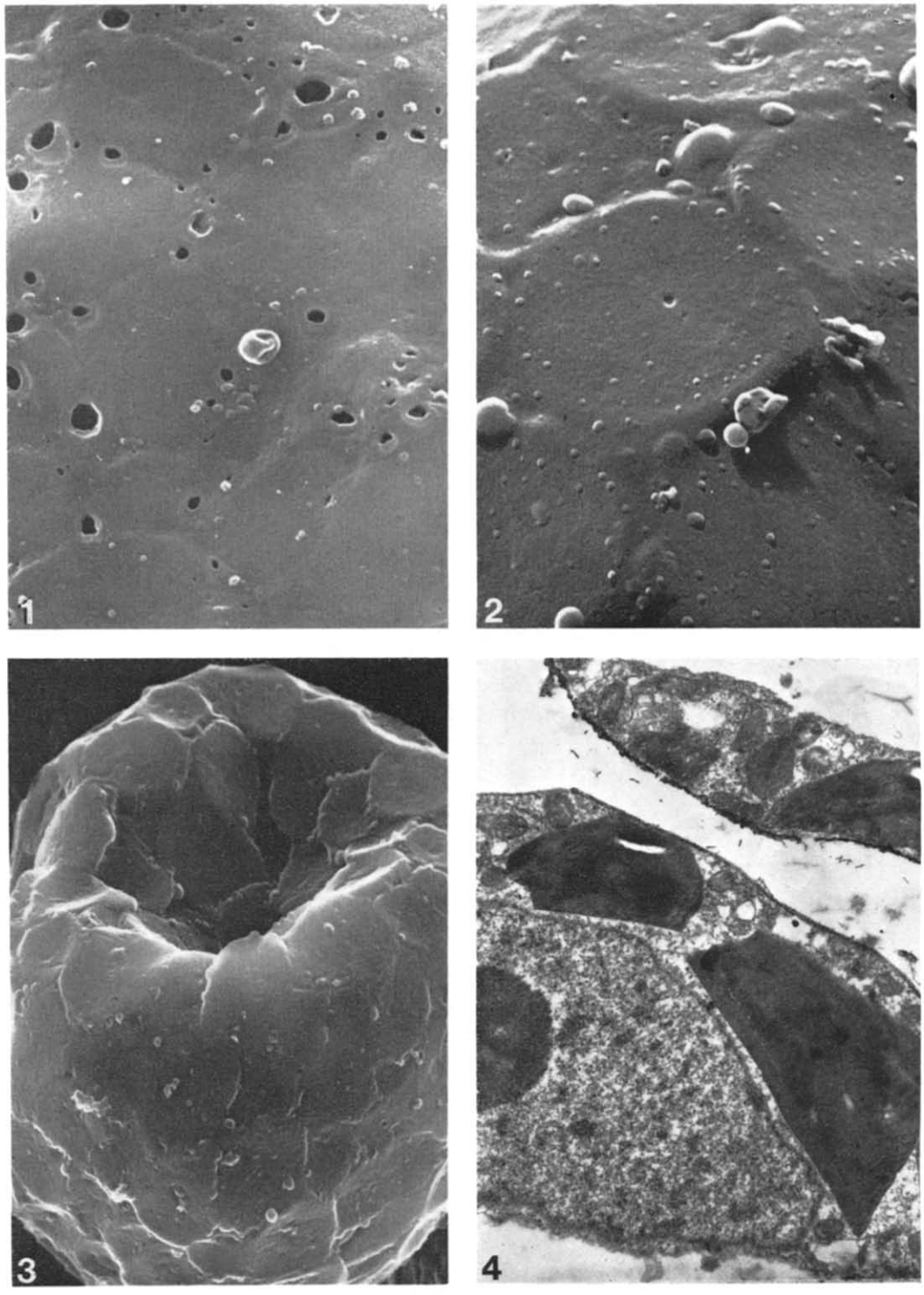
Such a technical advance requires for its consolidation a concomitant improvement in interpretative skills and criticism. In a recent paper Clark and Glagov (1976) have expressed disquiet concerning the interpretation of SEM images. The authors give examples from published work of evident misinterpretation of results and they lay down a set of guidelines for workers in this field. Unfortunately they omit a practical demonstration of the problems they discuss.

Our own interest lies in the surface properties of the plasmalemma of higher plants, made accessible by the preparation of isolated protoplasts. Protoplasts present a unique opportunity to study such problems as membrane mobility in the plant plasmalemma (Williamson *et al.*, 1976; Burgess and Linstead, 1977b), the interaction of membranes with viruses (Burgess *et al.*, 1973; Kubo *et al.*, 1976), the behaviour of membranes under the influence of fusing agents (Burgess and Fleming, 1974; Burgess *et al.*, 1977) and the formation of the plant cell wall (Burgess and Linstead, 1976, 1977a). In all these fields interpretation of the SEM image involves extrapolation from existing results obtained by the study of thin sections or replicas. This paper attempts to illustrate by practical example some of the problems encountered in making this extrapolation, and describes ways of overcoming them.

METHODS

Our standard conditions for the preparation of specimens of protoplasts for SEM have been described in detail elsewhere (Burgess *et al.*, 1977). Such deviations from this procedure as have been examined are described in the text (Results and Discussion).

In order to examine critical point dried or coated specimens as thin sections by transmission microscopy, the following procedure was adopted. Dried protoplasts were dusted onto the surface of a clean glass slide, and the slide placed in a vacuum coating unit. Carbon and gold coatings were then applied as in our standard proccdure (Burgess *et al.*, 1977). The coated protoplasts were then washed off the slide into a glass tube using dry acetone. This



Figures 1–4

was replaced by 1,2-epoxy propane and the protoplasts were then embedded in Araldite in the usual way. To study the effects of critical point drying only on internal structure, dried protoplasts were placed into dry acetone and embedded as before.

For the production of surface replicas, the following procedure was used. Protoplasts were fixed, critical point dried and coated with gold and then carbon on a glass slide. A stainless steel stub covered with double sided adhesive tape was then placed tape side down onto the layer of coated protoplasts. The protoplasts adhered to the tape. The stub was then coated with a thick layer of carbon only. This helped to reduce charging in the final specimen. Next, the protoplasts were broken by brushing the stub with a fine paint brush. The stub was then placed in sodium hypochlorite solution (BDH, Poole, 10–14% w/v available chlorine) for 30min. This removed loose debris and digested organic material covering the replica. After washing, the specimen consisted of fragments of the original carbon and gold coating embedded carbon side down on the surface of the adhesive tape. The SEM image of such a specimen is therefore produced by electrons impinging upon the inner surface of a gold layer formed in contact with the outer surface of the protoplast. In SEM examination of whole protoplasts the image is formed from the outer surface of a gold layer which is itself overlying a carbon layer in contact with the protoplast surface (see text).

The binding of tobacco mosaic virus (TMV) to the protoplast membrane was carried out using the procedure for cowpea chlorotic mottle virus previously published (Motoyoshi *et al.*, 1973), except that the concentration of TMV was 50μg/ml and the concentration of poly-L ornithine was 10μg/ml. Under these conditions high rates of binding are obtained, facilitating the identification of the virus. These conditions, it should be noted, do not represent the optimum for actual virus infection of tobacco protoplasts with TMV.

RESULTS AND DISCUSSION

Specimen preparation

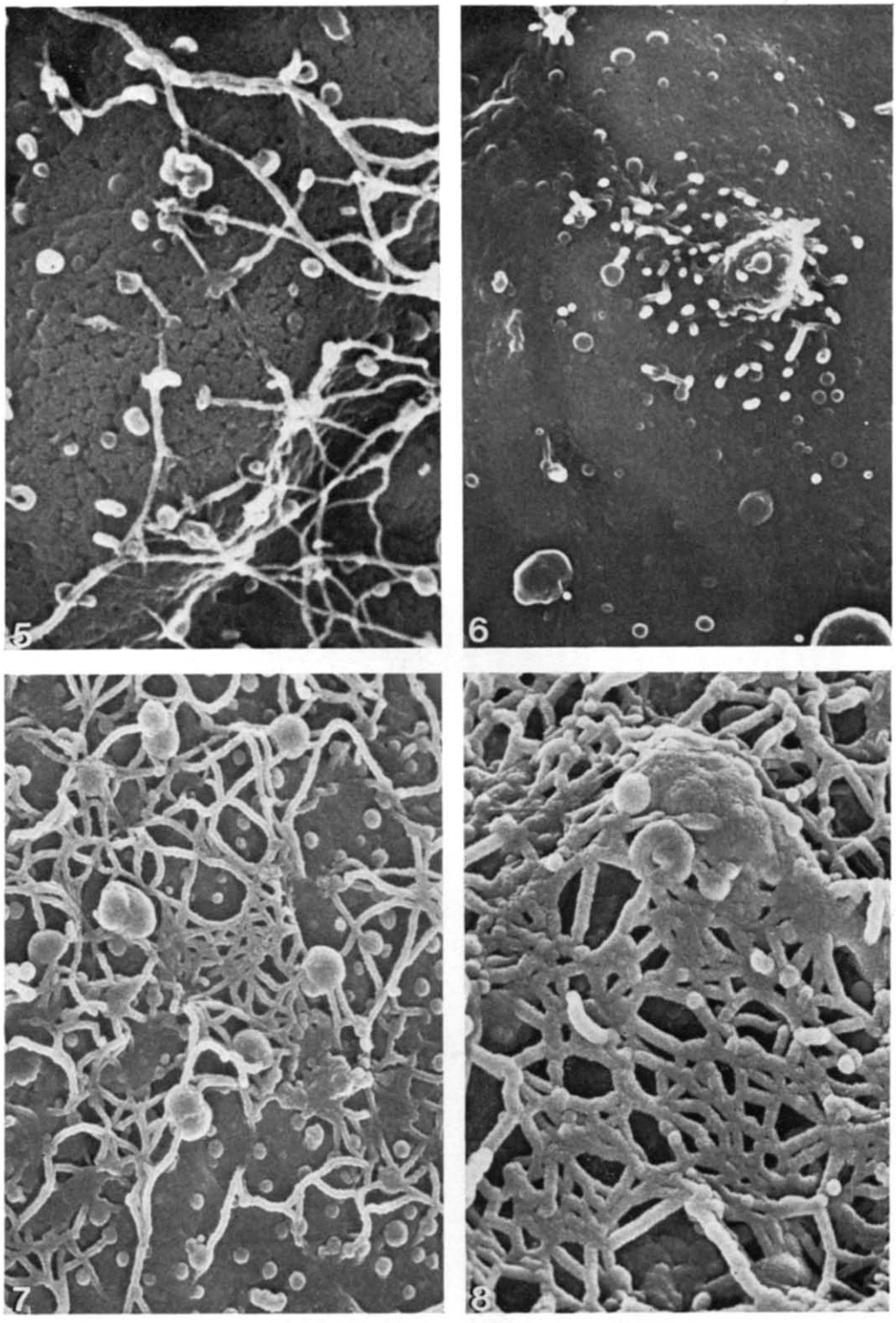
Many specimens which are now being examined by SEM have been previously studied using thin sections in the transmission mode. In these circumstances it is desirable that established regimes for fixation and dehydration be maintained as closely as possible. If this is done it is reasonable to assume that the standard of preservation of the SEM specimen will match the known standard from thin section study. It may be tempting, for example, to omit secondary fixation with osmic acid, on the grounds that a major benefit of this is to enhance electron contrast in transmission microscopy. However, a comparison of plant protoplasts prepared by double fixation and fixation with glutaraldehyde alone shows that omission of the osmic acid stage results in a profound change in appearance of the surface membrane (Figs. 1, 2). The membrane fixed in glutaraldehyde alone shows the presence of large numbers of holes, which are clearly artifactual. This gross difference in the appearance of the membrane serves to emphasise that correlation between the structure of specimens fixed in different ways is liable to be misleading. Time schedules should also be adhered to; protoplasts which have been dehydrated at an accelerated rate show a

Fig. 1. Part of the surface of a tobacco protoplast fixed in glutaraldehyde alone after 4½hr incubation in medium. The specimen was critical point dried, and coated with carbon and gold whilst being rotated. The membrane is extensively holed. ×12,000.

Fig. 2. Part of the surface of a specimen similar to that shown in Fig. 1, but fixed with osmic acid after glutaraldehyde, and held stationary during the coating procedure. The surface is intact with only minimal shrinkage onto the underlying chloroplasts. ×12,000.

Fig. 3. A tobacco protoplast, double fixed but dehydrated rapidly through an alcohol series. The outline of cytoplasmic chloroplasts is very clear due to shrinkage or collapse of the plasmalemma during the drying process. ×4500.

Fig. 4. Section through part of two tobacco protoplasts which were critical point dried and coated with carbon and gold prior to embedding. Cytoplasmic components are clearly identifiable, and the integrity of the nuclear membrane and the tonoplast has been maintained. The coating material can be seen at the outer plasmalemma surface. ×8000.



Figures 5–8

characteristic polygonal outline due to the collapse of the membrane on to the underlying peripheral chloroplasts (Fig. 3). Rates of dehydration as a source of poor preservation for SEM have been discussed by Hughes *et al.* (1976). The use of direct dehydration methods such as 2,2-dimethoxy propane (Johnson *et al.*, 1976) have little to recommend them in the absence of parallel trials to establish their efficacy using thin sections.

In the case of specimens which have not been previously examined, it is clearly desirable to confirm by independent means that the fixation and dehydration methods used are capable of giving adequate preservation of ultrastructural detail. Examination of thin sections by transmission microscopy should suffice for this, since a considerable consensus already exists as to what constitutes 'good fixation'. Only in a few specialised cases such as pollen grains or other specimens not requiring extensive preparative handling prior to their examination in the SEM is it acceptable to forego such an examination.

Preparation of SEM specimens involves an additional stage which is absent from the normal dehydration/embedding sequence for transmission work. This is the drying of the specimen to allow it to be inserted into the microscope. Customarily this is done by substitution of a dry organic solvent, usually amyl acetate, with another liquid, commonly carbon dioxide, and the removal of this secondary liquid above its critical point. This procedure was advocated by Anderson (1951), and its superiority to air-drying or freeze-drying is widely accepted (Meller *et al.*, 1973). The procedure introduces variables in the form of the two extra solvents, and the pressure required for the critical point of the secondary liquid to be exceeded. The effects of these can be checked by embedding the specimen after critical point drying, and examining thin sections in the normal way. When this is done with protoplasts the degree of structural preservation retained within the cytoplasm is remarkable (Fig. 4).

Coating procedures

For successful image formation without charging, it is necessary to coat the specimen with a conducting layer, usually of carbon followed by a heavy metal or alloy. Carbon coating alone gives rise to poor emission of secondary electrons and a low contrast image. The use of a metal such as gold on its own allows satisfactory emission but the quality of the image is degraded by the structure of the metal coating layer (Fig. 5). The use of alloys improves this situation, but double coating is nevertheless more acceptable as a general procedure.

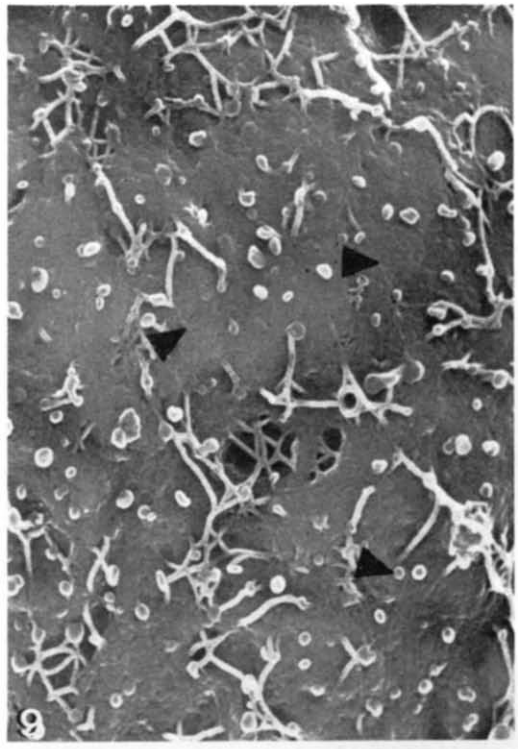
Coating has immediate consequences for the interpretation of fine detail at or near the specimen surface. In the case of fibrous or elongated structures standing away from the surface, the principal effect is to increase their apparent size (Burgess *et al.*, 1977). In this way fine fibres of, for example, cellulose, may be built up to a point where they are easily resolvable by SEM. A secondary effect of coating is that all elongated structures such as cellulose fibres or rod-shaped viruses take on a hollow appearance. This is due to the image-forming electrons originating from the surface of the structure and may not be interpreted as

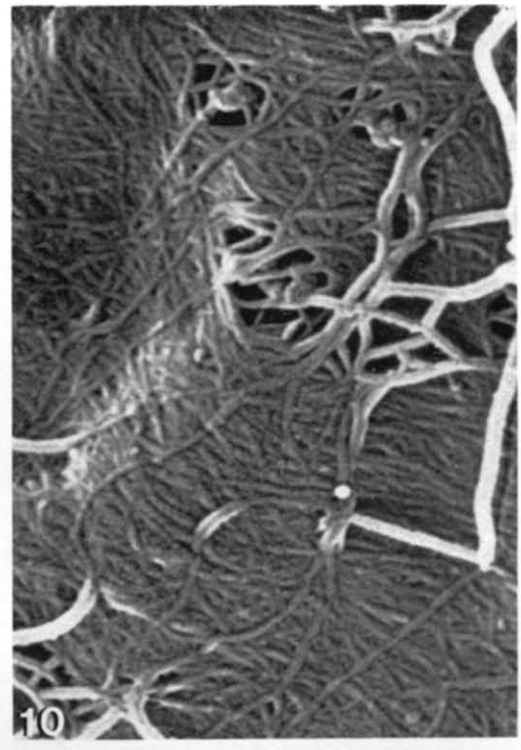
Fig. 5. Part of the surface of a tobacco protoplast during early wall regeneration. The specimen was double fixed, critical point dried using carbon dioxide, and coating with gold only. The image is coarse and the structure of the gold coating clearly visible on the membrane surface. ×17,000.

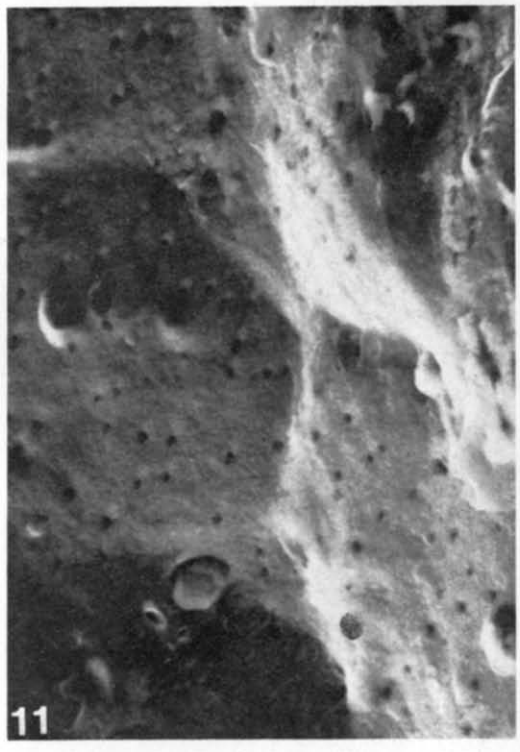
Fig. 6. A group of tobacco mosaic virus particles at the surface of a tobacco protoplast. The rods appear to be hollow due to the reflection of electrons from the surface coating of carbon and gold. ×12,000.

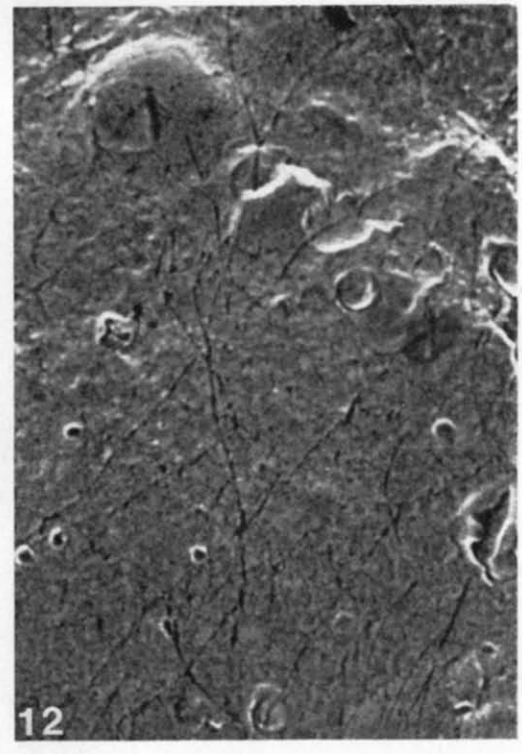
Fig. 7. Part of the surface of a regenerating tobacco protoplast. The specimen has been coated with carbon and gold in the usual manner. In places the fibres seem to approach one another to give a 'filled in' appearance. ×13,000.

Fig. 8. Part of the surface of a protoplast from the same population as Fig. 7, fixed and processed identically, but with an exceptionally heavy carbon coating prior to gold. This figure is printed at the same magnification as Fig. 7. The fibres appear to be much thicker, and the general impression is of a more mature and 'filled in' stage of the young wall. ×13,000.









Figures 9–12

indicating 'hollowness' even when, as in the case of TMV, other microscopical methods show this to be the case (Fig. 6). An incidental artifact of coating is that the thickness of fibres and rods depends upon the thickness of the coating used. This is clearly shown in the case of the regenerating wall around protoplasts (Figs. 7, 8). Qualitative differences may also emerge as a result of varying the amount of coating; thus in the case of the wall shown in Fig. 8, 'filled in' areas are more common than in the same specimen prepared with a lighter coating (Fig. 7).

A more serious problem may arise with small details close to the surface of a membrane, or on the surface. Here the process of coating the specimen may serve to obscure fine detail completely and thus present a misleadingly simple image in the SEM. This has been exemplified in our own studies by the appearance of the colloidal gold/Concanavalin A complex (Burgess and Linstead, 1977b). In the scanning microscope the complex is seen as a particle of irregular shape and in a range of sizes. When specimens which have been coated for SEM are subsequently embedded in resin and sections examined by TEM, it is clear that a particle which would appear as a single unit in SEM in fact comprises several particles of the gold colloid. It is likely that in some circumstances fine details close to surfaces might be completely obscured by a layer of coating material. In the case of the regenerating wall around isolated plant protoplasts, many areas of membrane contain fibres which by their spatial relationship appear to be continuous, although the continuity is seen only at reduced contrast or not at all (Fig. 9). The apparent size of a structure may also depend upon its relationship to the surface. In the case of a coated wall around an isolated tobacco leaf cell fixed prior to treatment with cellulase (Fig. 10), the fibres lying in the surface of the exposed wall appear to be thinner than fibres which protrude away from the surface, although in some cases they are clearly continuous and thus likely to be of uniform thickness. This represents a severe limitation on the interpretation of the surface structure of coated specimens by SEM.

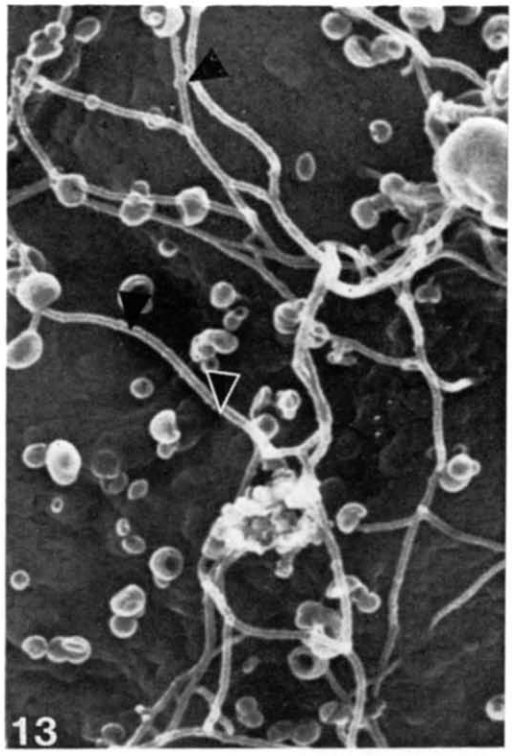
In an attempt to overcome these artifacts of coating, we have examined a simple replica technique which allows the inside surface of the coating layer to be examined by SEM (see Methods). By the use of this technique the same machine may be used in the same mode to view the outer and inner surfaces of the coating layer and thus allow a direct comparison of the structures observed to be made. Fig. 11 shows the inside surface of the coating layer from a specimen identical to that shown in Fig. 2. The small projections which are visible at the outer surface of the coating layer are seen as small depressions in the inner surface. No fibres are visible. When the inner surface of a layer coating a regenerating protoplast is examined (Fig. 12) the fibres at the surface are observed as depressions in the coating layer. These depressions are considerably narrower than the diameter of coated fibres above the surface (Figs. 7, 8). Furthermore, their thickness is independent of the amount of coating material used and therefore is a truer representation of the structure to which they correspond. Large and small depressions are also visible, corresponding to the large and

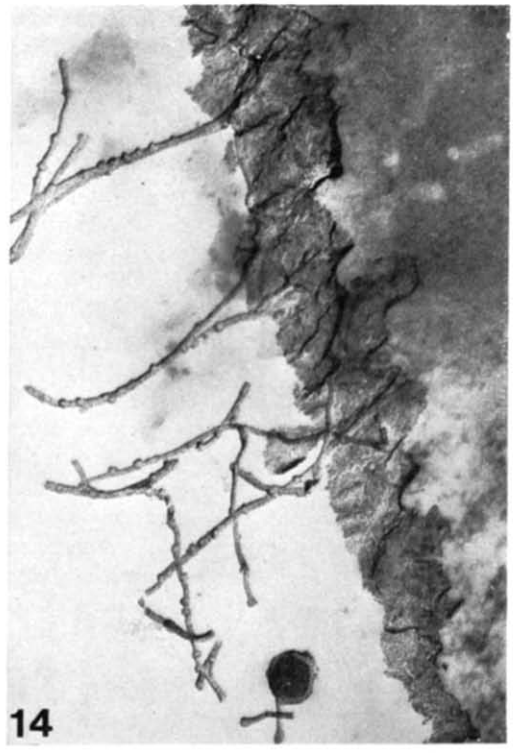
Fig. 9. Part of the surface of a tobacco protoplast during wall regeneration. The clarity with which fibres are rendered depends on their position relative to the underlying membrane. In several places (arrows) clear fibres are continuous with fibre traces. The traces probably represent fibres which are lying very close to the plasmalemma. ×13,000.

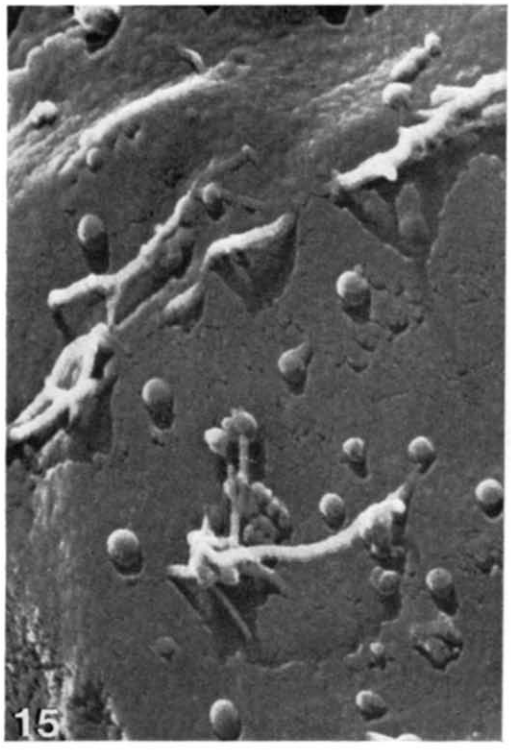
Fig. 10. The surface of a tobacco leaf cell prior to cellulase treatment. The fibres comprising the wall appear to be of different thicknesses. Those standing away from the surface have received more coating material and so appear to be thicker than the fibres within the surface. ×15,000.

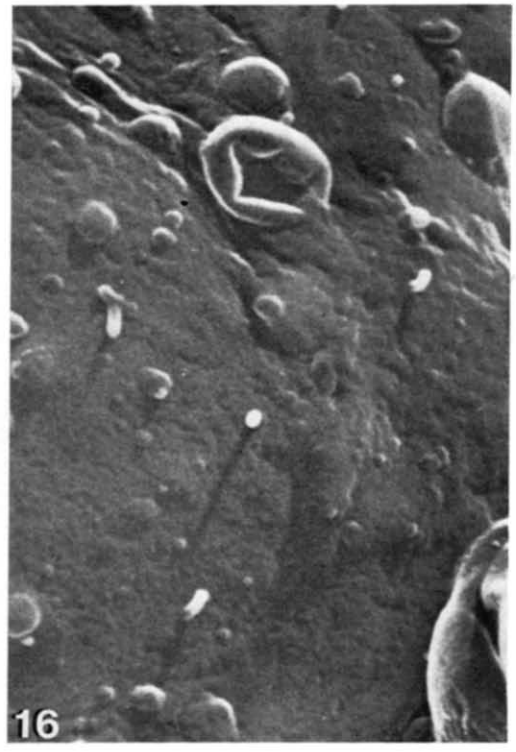
Fig. 11. The inside surface of a replica from a protoplast fixed after 4½hr incubation. The small depressions in the replica correspond to the small projections seen when the protoplasts are scanned in the normal mode (cf. Fig. 2). ×15,000.

Fig. 12. The inside surface of a replica from a protoplast which was regenerating a wall at the time of fixation. The traces of wall fibres are visible as depressions in the replica. The diameter of these depressions is independent of the amount of coating material used in the production of the replica. ×30,000.









Figures 13–16

small projections present in the outer surface view obtained by examining whole coated protoplasts. This technique represents a useful method of assessing the degree to which the size, and indeed the presence, of a structure in the normally scanned specimen is an artifact of the coating procedure used.

Identification of new structure

The SEM image by its mode of formation is liable to present information which is quite novel when previous studies on a particular material have been limited to thin section examination. In biological systems, this in fact means that surface views of membranes are obtained where previously their structure had been inferred from TEM of transverse sections or replicas, perhaps corroborated by limited serial sectioning and fortuitous views across small areas of surface as glancing sections. The ability to view a large area of surface is particularly useful in describing the distribution of a surface related effect such as lectin binding or cellulose synthesis (Burgess and Linstead, 1976, 1977a,b). Coincident with such studies however, is the probability of visualising entirely new and unexpected modulations of the surface. This results not only from the mode of image formation in the SEM, but also simply from the ability to examine large areas of surface. It is a matter of common experience to all electron microscopists, particularly in thin section work, that sampling error is a serious problem. Transient and surface related phenomena are liable to be entirely missed or at least underrated in importance. SEM may therefore give a quite unexpected view of the complexity of surfaces. The difficulty in interpreting such a view consists of attempting to confirm its validity by some other technique. An example of this is the transient change at the protoplast surface which appears to precede cell wall regeneration (Burgess and Linstead, 1976). Prior to the appearance of fibres the surface becomes covered with small projections, some of which apparently relate to new fibre formation. The presence of such projections has been confirmed independently by the use of replicas (Williamson *et al.*, 1977). The relationship between the fibres and the projections may be circumstantial however, and this requires further clarification. The loss of surface detail due to the coating referred to above sets the limit to the information which SEM can give in this circumstance. Another example of new structure which appears in SEM in this system is the presence of small particles along the length of coated cellulose fibres (Fig. 13). Here the novelty is presumably due to the coating forming over a component which is not stained by the heavy metals used in thin section work. Sections of coated specimens confirm the presence of the irregularities (Fig. 14). This illustrates a further hazard in the correlation of structure seen in SEM and in sections; the former gives a picture based on the ability of a structure to support the coating materials used, whilst contrast in the transmission microscope is generated by chemical or physical binding of electron dense metals such as osmium, uranium and lead.

Three-dimensional effects

In thin section work, information concerning the three-dimensional structure of a specimen or organelle can be gained by serial sectioning.

Fig. 13. Part of the surface of a tobacco protoplast during early wall regeneration. Some of the fibres show the presence of particles or irregularities in thickness along their length (arrows). ×15,000.

Fig. 14. A section through the surface of a protoplast similar to that shown in Fig. 10. The specimen was critical point dried and coated with carbon and gold prior to embedding. The local irregularities in thickness of the cellulose fibres is clearly shown. ×15,000.

Fig. 15. Part of the surface of a tobacco protoplast during early wall regeneration. The specimen was coated with carbon and gold whilst stationary. The brightest fibres are clearly standing proud of the membrane, as shown by the shadows they cast. ×30,000.

Fig. 16. Part of a tobacco protoplast after exposure to tobacco mosaic virus particles. Unidirectional coating reveals the presence of the rods more clearly and also suggests that many are attached by one end to the surface. ×30,000.

This is a tedious technique involving considerable investment in effort and skill to provide significant information. SEM may yield such additional information by stereo-pair imaging or on some machines simply by manipulation of the specimen stage. Where translation of the specimen is only possible in two dimensions, uni-directional shadowing of the specimen represents a simple modification to standard technique and may assist greatly in image interpretation. Surfaces are thrown into relief and become more easily understood as three-dimensional units. In particular, projections from surfaces cast a shadow after uni-directional shadowing which may be informative and also diagnostic. Figure 15 shows this effect with early wall fibres on a regenerating protoplast. It is clear that many of the fibres stand quite clear of the membrane. In the case of TMV (Fig. 16) the shadow cast by the virus shows that it is attached by one end to the membrane, and incidentally allows more confident identification. With rotary shadowing such a particle appears merely as a bright spot on the surface (Fig. 6).

GENERAL CONCLUSIONS

In their critical paper on the evaluation and publication of scanning micrographs Clark and Glagov (1976) have suggested four steps to be taken by authors to avoid misinterpretation of SEM results. These were the confirmation of new structure by some independent technique, the elimination of 'aesthetically' pleasing pictures which contribute nothing of value, the recognition and description of artifacts and the full publication of techniques. These are sound guidelines.

The problem is essentially one of the use to which SEM is now put. Formerly, as is the case with any new technique, the results which were obtained were comparatively unambitious reworking of old ground in a novel way. With higher resolution instruments, SEM has now reached the stage where it can make a unique contribution in areas where its use is appropriate. These are primarily in the study of surface phenomena. For useful progress to be made it is essential that the pitfalls inherent in the technique should be recognised both by authors and reviewers of manuscripts. It is hoped that the illustrations given in this discussion will contribute to a greater awareness of interpretative traps and the possibility of avoiding them.

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