OPTIMUM PREPARATION, PRESERVATION, AND PROCESSING TECHNIQUES FOR GRAPTOLITE ELECTRON MICROSCOPY

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ABSTRACT. Published guidance on the preparation of ultrathin sections of biological material rarely gives sufficient detail to enable fossil material in general and graptolite material in particular to be prepared to a consistently high standard. The crucial steps relative to pre-microtoming, ultramicrotoming, and post-microtome work, including museum storage, are described together with the 'tricks of the trade' that, taken in total, result in successful micrographs.

TRANSMISSION ELECTRON MICROSCOPY (TEM) has been an important technique in biological and medical science for many years but its application to fossil material poses rather different problems and such material is generally more difficult to prepare. Urbanek (1978) suggested that ultrastructural research may help to resolve some of the problems highlighted by earlier morphological and phylogenetical studies on graptolites. Wide discussion of the mechanism of periderm secretion in graptolites has suggested that TEM studies may aid a better understanding of the relations between inferred soft parts and the skeletal material.

Williams (1965, pp. H254–H255) and Nye *et al.* (1972) described a technique which may be used simultaneously to section both hard and soft tissues for study by reflected light, a technique which may be applied, for example, to recent brachiopods. The initial stages of impregnation with epoxy resins, described by Nye *et al.* (1972), are similar to those which we have used in the preparation of graptolites for TEM.

The use of ultrathin sections in palaeontology has been slowly adopted, largely because of the potential preparation difficulties involved and partly because of costs. Diamond knives commonly exceed £1000 sterling and are not much cheaper to have sharpened. In contrast, stereoscan electron microscopy (SEM) is now used routinely in most groups and especially so in graptolites (Rickards *et al.* 1982). There is no logical reason why TEM studies should not become equally routine, for they are a necessary complement to SEM work, contributing towards our understanding of skeletal morphology, as has been confirmed by recent studies of graptolites (e.g. Crowther 1981). This paper describes how to isolate graptolites from the original matrix, and their subsequent preparation for scanning and transmission electron microscopy. Photographic techniques employed to obtain maximum contrast in the final micrograph are then described briefly and relevant problems of museum documentation discussed.

Graptolite material is quite variable in its preservation of ultrastructural detail, sometimes exquisite but often showing varied forms of degradation. Even the best techniques, coupled with infinite care, occasionally give barely adequate results, whilst some specimens seemingly respond well to very primitive technology (Berry and Takagi 1970). The current procedures evolved during tenureship by the authors of a Research Grant from the Natural Environment Research Council on graptolite ultrastructure, although considerable progress was made in the 1970s and summarized by Crowther and Rickards (1977). A number of the pre-sectioning techniques have not been described before but some were developed many years ago by Professor O. M. B. Bulman and by the junior author.

[Palaeontology, Vol. 28, Part 4, 1985, pp. 757–766.]

CHEMICAL ISOLATION

Most SEM and TEM work is enhanced by using specimens chemically freed from the rock matrix. Adhering rock or pyrite can seriously damage diamond knives; but for SEM studies, graptolites in the matrix have been used successfully (Rickards *et al.*, 1971; Crowther and Rickards 1977) in part to test the effect of chemical preparation on the ultrastructural detail, which appears to be negligible.

The procedure for chemical isolation follows two basic routes depending upon whether the matrix is highly calcareous or not. It should always be carried out in a fume cupboard. Many limestones containing graptolites can be immersed as small pieces (a few cm across) in dilute hydrochloric acid (10 % HCl; text-fig. 1), in glass beakers, releasing the graptolites often in a matter of hours. There is no way of determining in advance whether the periderm is strong enough to withstand the effervescence, or whether the graptolites may be isolated by any method, other than by trial and error. If the effervescence appears to be breaking up the emerging specimens, then the acid may be diluted to slow down the action, or a non-effervescing or less violently effervescing acid (such as acetic acid, CH_3COOH) can be used.

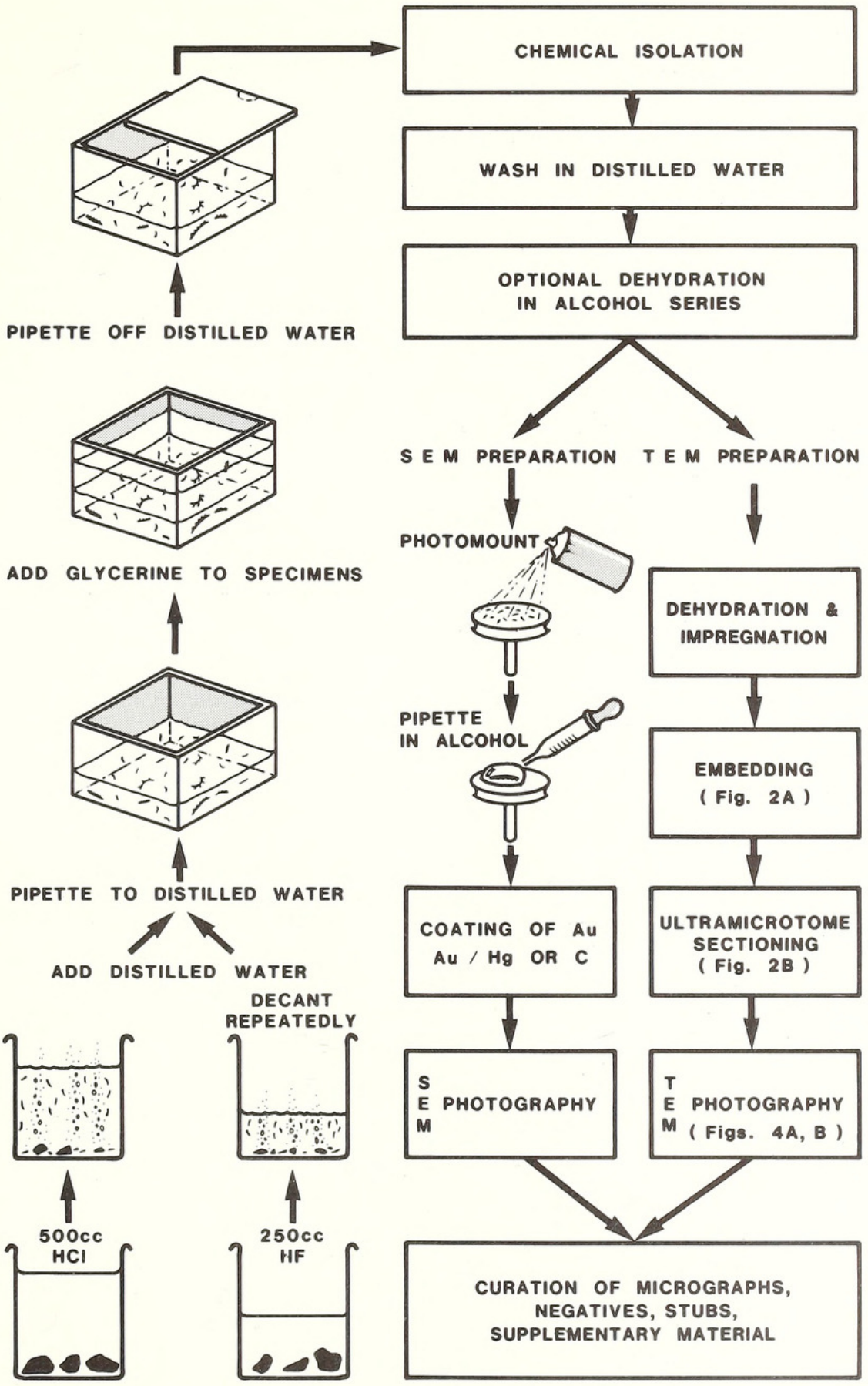
A second procedure may be used when the terriginous content of the limestone is so great that a relatively firm rottenstone remains, failing to release the graptolites. When this happens the blocks must be treated with 10 % HCl for up to three weeks, changing the acid daily, until all possible $CaCO_3$ has been removed. At that stage the rottenstone can be treated with 60% hydrofluoric acid, in polythene beakers, which usually releases the graptolites within a few hours. In all the above treatments, two or three pieces of rock can be placed in up to 500 cc of HCl or up to 250 cc of HF (text-fig. 1), or the whole process can be made into an assembly line using large containers and quantities of fluid.

The next stage is the most laborious and critical if the full suite of isolated specimens is to be retained. Some specimens will have floated in an oily scum, often adhering to the meniscus. These need to be carefully pipetted off, using either glass or polythene hand pipettes (for HCl or HF respectively), into a container of distilled water in which they may still float. The remaining fluid is then carefully decanted and washed away using copious supplies of water from the fume cupboard taps. Specimens 'floating' in mid-fluid can be pipetted out at this stage. As a rule, a majority of specimens lie in the muddy debris at the bottom of the beaker. Distilled water should be added to the beaker, stirring the sunken specimens and mud, and the whole of the above decanting process repeated several times until the acid has been washed out. Pipetting can take place at the same time but eventually the whole remaining mass of mud and graptolites needs to be carefully decanted into a wide, shallow, preferably white-bottomed picking dish. At no stage should the specimens be allowed to dry out or to support their own weight in air, for many collapse, especially if the original rock came from a tectonized region. However, the most delicate rhabdosomes may be supported by fluid and can easily be held in that medium until they are transferred to the long term resting medium, namely viscous glycerine.

Initial picking by hand pipette is from the picking tray into distilled water in glass beakers. The graptolites must not be stored in distilled water for longer than about twenty-four hours, since this encourages the growth of fungus. The main problem at this stage is the number of fragments and early growth stages that may be masked by the mud and missed altogether. Specimens should be washed in several changes of distilled water for a few hours.

The best way to transfer delicate specimens into viscous glycerine is to reduce the amount of distilled water over them by pipetting it off until only a few mm cover the specimens. Then glycerine is added gently around the margins of the container, thus pushing the specimens away from the sides. Eventually, glycerine can be dropped gently on top of them, weighing them down (text-fig. 1). The remaining distilled water floats above the glycerine and, when it is completely separated from the graptolites, can easily be pipetted off to leave the specimens in a firm, supportive, fluid matrix.

Specimens that floated on initial extraction may sink quite quickly; if not, they will have tiny gas bubbles lodged inside the thecae. These can be removed by pipetting the specimen directly into alcohol, a somewhat violent process which may snap the specimens; alternatively they can be cooled



TEXT-FIG. 1. Flow chart summarizing preparatory stages for storage, SEM and TEM work.

in a refrigerator when the small gas bubbles may escape (dishes of alcohol should not be placed in a refrigerator without proper consultation with a laboratory technician, for there is a risk of explosion). Once the specimens have sunk, transfer to glycerine can proceed as above, and the graptolites are now ready for the preparatory stages described below, leading to electron microscopy.

As an important aside, it should be mentioned that in preparing glycerine held specimens for light microscopy (as, for example, in the production of slides or resin mounts; Hutt and Rickards 1967), any chemical clearing using Schultz' Solution ($KClO_3$ and HNO_3) should be done in the absence of glycerine since there is a slight risk of accidentally producing TNG or TNT.

SEM PREPARATION

Preparation of graptolites for use in the SEM involves mounting the specimen on a stub and subsequently coating it, preferably with gold; other coatings may be tried, such as gold/palladium or carbon, and occasionally it may not be necessary to coat at all if much pyrite adheres to the specimens. The specimen is pipetted from glycerine into absolute alcohol, in which it is washed for approximately thirty minutes. An intermediate distilled water stage has proved unnecessary. The stub is then sprayed with a very thin film of 'Photomount' (see Crowther and Rickards 1977), or double sided adhesive tape may be used, and the specimen is pipetted with a drop of alcohol to the surface of the stub. The graptolite may be oriented at this stage by manœuvring it with a damp hair, although great care must be taken with very fragile specimens. The alcohol is then allowed to evaporate. Finally, with the Sedgwick Museum material, the stub is placed in an 'EMscope' sputter coater and coated with 200–500 Å of gold, after which it is ready for examination in the SEM.

TEM PREPARATION

The specimen is pipetted from glycerine into a vial containing distilled water and washed in three changes of water for fifteen minutes each. Commonly, a graded series of ethyl alcohol dilutions is then used for dehydration of the specimen but it is possible to use three changes of absolute alcohol (about ten to fifteen minutes each). At this stage the specimens may be stored indefinitely in the alcohol. After dehydration the specimens are soaked in a 50/50 mixture of propylene oxide (1,2—epoxy propane) and absolute alcohol and then in a further two changes of 100 % propylene oxide for fifteen minutes each. This facilitates uniform impregnation of the specimen by the epoxy.

During each of the above stages the vial is placed in a rotator so that the liquid circulates freely inside the specimen. Whilst the specimens are soaking in the propylene oxide the epoxy resin mix may be prepared. Propylene oxide should be used in glass rather than plastic containers and only in a fume cupboard because of its toxicity, volatility, and flammability. After use it should be flushed away with continuous water flow for five minutes.

Embedding

The 'Agar 100' embedding kit (equivalent to 'Epon 812') is suitable for embedding graptolite material. The 'Epon' mixture is blended and accelerator added just before use. A graduated cylinder, a small (100 ml) conical flask, and the containers of resin and hardener are warmed in an oven at 60 °C. The four components, resin, DDSA hardener (dodecynl succinic anhydride), MNA hardener (methyl nadic anhydride), and BDMA accelerator (N benzyl N–N dimethylamine) are mixed by pouring them in turn into the graduated cylinder. Before adding BDMA the other three components should be stirred thoroughly since direct mixing of BDMA and MNA may be explosive. The mixture is then poured immediately into the warm conical flask and stirred for about one minute. A few air bubbles may develop but these will dissipate if the mixture is allowed to stand for a short time at 60 °C. Epoxy resins, hardeners, and accelerators should be handled with care in a fume cupboard.

Difficulties may be experienced in obtaining blocks of the correct hardness for ideal sectioning. The following mixture has recently been used successfully to embed specimens of *Monograptus formosus* from the Mielnik borehole in Poland: resin 25 ml; DDSA 11 ml; MNA 14 ml; BDMA 1 ml. The

hardness of the final block may be controlled by varying the proportions of DDSA and MNA in the resin mixture. When proportions of DDSA to MNA were tried in the quantities 12·5/12·5 ml the resultant blocks were too soft, and at 10/15 ml they were too brittle. Hardness may also be increased as the concentration of the accelerator (BDMA) increases, but the block may become brittle and difficult to section as a result.

After the resin components have been stored for about five months, they begin to give inconsistent results. It is suggested that the accelerator should be stored in a dessicator in the dark. Several workers have found that the accelerator for 'Spurr' resin, for example, has a very short shelf life (about two weeks). The liquid epoxy resin is soluble in absolute alcohol and all glassware should be rinsed in alcohol after use.

The specimen is then transferred in propylene oxide to a small tray (a vial top or petri dish is ideal) and an equal volume of epoxy resin mixture is added. These are left for one hour, loosely covered to prevent evaporation. The covers may then be removed and the specimens left in a fume cupboard overnight, during which time the propylene oxide will evaporate. They are then polymerized in a thermostatically controlled oven in three stages (35 °C, 45 °C, and 60 °C), over a period of thirty-six hours: overnight at 35 °C, next day at 45 °C, and overnight at 60 °C. The maximum internal temperature of the epoxy resin obtained during polymerization will affect the properties of the resultant block. If the temperature is too low, the block will be too soft; if the temperature is too high, bubbles may form in the epoxy and the block will be too brittle (Nye *et al.* 1972). Ideally the end product should be an amber colour and of moderate hardness (i.e. will not deform when pressed with a finger nail).

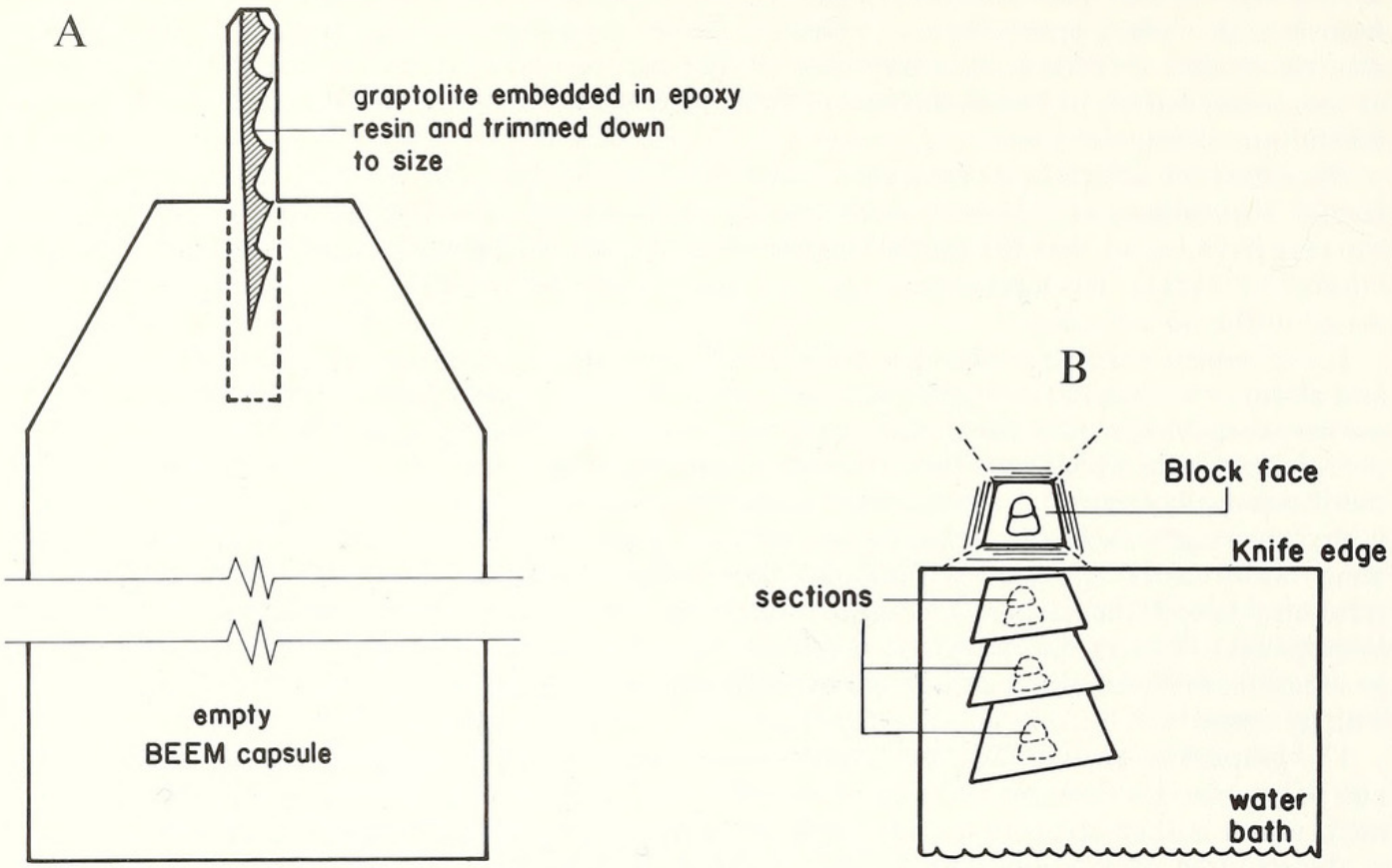
The graptolites are now ready for cutting into blocks and mounting on 'Araldite' stubs made and polymerized in the same way and at the same time. The hardened resin is trimmed from the specimen, which is then stuck to a stub (made in polyethylene capsules known as 'BEEM capsules') in the required orientation, with 2-tube 'Araldite'. The graptolite may be trimmed down to size with a heavy duty, backed razor blade. This step is carried out with the specimen held under water because it is usually too small to be clamped and consequently is easily lost during trimming in air.

Where a series of transverse sections from a specimen of three or more thecae in length is required, it is suggested that a small hole be made in the end of a BEEM capsule and the trimmed specimen then forced through the hole so that the end to be sectioned protrudes from the capsule (text-fig. 2A). The capsule is then filled with liquid epoxy resin and polymerized as before. The specimen will have been polymerized twice but this does not adversely affect the end-product. When specimens of this type were simply stuck on stubs with 2-tube 'Araldite', they often snapped off during sectioning on the ultramicrotome.

Cutting ultrathin sections

After polymerization the block is held in the clamp of the ultramicrotome and the area around the graptolite trimmed into a pyramidal shape (text-fig. 2A). The face to be sectioned should be cut until it reaches the specimen to save time and wear on the knife edge. The block face is shaped so that cut sections will form a ribbon, perpendicular to the knife edge, on the surface of the water bath (text-fig. 2B). It is therefore necessary for two edges of the block to be parallel to each other and orientated in the ultramicrotome so that they are parallel to the knife edge (text-fig. 2B). It is best if one side is longer than the other, forming a trapezium orientated such that each new section pushes along the whole width of the previous one, so detaching it from the knife edge. Neatly rectangular slices do not form such a reliable and straight 'ribbon'.

A diamond knife is held in the knife holder, tilted at approximately 4°, and the water bath is filled with distilled water until it appears white in polarized light (i.e. maximum reflection is obtained with a 'flat' meniscus). The knife is advanced manually towards the block until almost touching it, as seen through the binocular microscope, then locked in position. It is often difficult to see just how close the block is to the knife edge. A mirror placed under the specimen will reflect bright, white light between the knife edge and block face, making it easier to judge the gap between them. The knife should never



TEXT-FIG. 2. A, showing how embedded graptolite (three or more thecae in length) may be orientated for transverse sectioning; the encased specimen is forced through a hole at the shaped end of the BEEM capsule which is then filled with epoxy resin and polymerized (full explanation in text). B, sketch of trapezium-shaped block face which causes the cut sections to form a saw-edged ribbon on the surface of the ultramicrotome's water bath.

touch the block face at the start since this will undoubtedly damage the edge. When the knife and block face are as close as possible the block is advanced 0·5 μm at a time until a complete section is cut from the face, after which the block is advanced automatically by a set thickness.

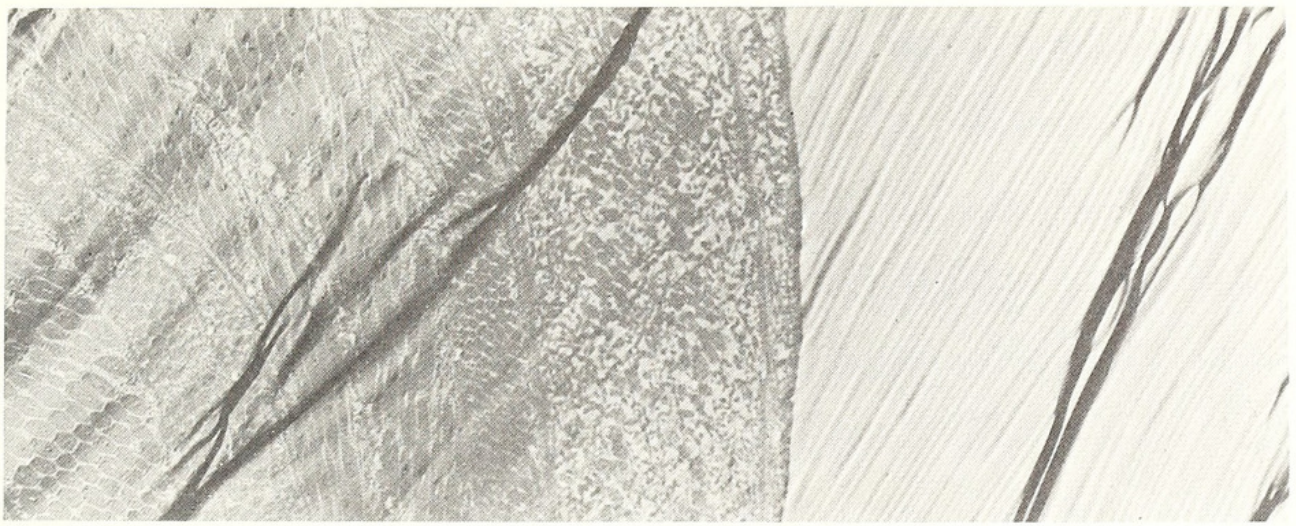
Interference colours are used to estimate the thickness of cut sections. Peachey (1958) provided a correlation between section thickness and interference colours as follows:

Colour	*Thickness* Å (1 nm = 10 Å)
grey	600
silver	600–900
gold	900–1500
purple	1500–1900
blue	1900–2400
green	2400–2800
yellow	2800–3200

Ideally sections should have grey interference colours, especially if they are to be used for high resolution work. However, silver and occasionally pale gold sections have been used successfully for work at low magnifications. The position and the meniscus level of the water bath may have to be finely adjusted for optimum viewing of the interference colours. The ultramicrotome is very sensitive to touch and atmospheric conditions. It is therefore necessary to avoid contact with it as far as possible and also to eliminate any draughts in the room, especially those caused by doors opening; a steady temperature of 20 °C should be maintained. A cutting speed of 1·1 mm/s used with a knife angle of approximately 4° provides good sections of graptolite material. In general, hard specimens

are best cut at slower speeds than soft specimens. The optimum settings for all controls can only be realized by experimentation. Sections should float off evenly in a straight line and be of uniform colour, flat, and with no corrugations.

Most of the difficulties in cutting good sections arise from faults with embedding and the knife edge. For a further detailed discussion of faults observed in ultrathin sections, and their possible causes, see Reid (1974). Generally glass knives do not provide such good sections of graptolites as diamond knives, but they may be used to trim specimens prior to sectioning with the diamond knife. When viewed through the binocular microscope the edge of a glass knife can often be seen to crumble after only one cut of the material. When the embedding medium is softer, several cuts are possible but problems with corrugations within the section and the mounting medium may be encountered (text-fig. 3). After a few sections have been cut with a glass knife the edge becomes blunt and it is necessary to move to an unused part of the knife. Diamond knives are more durable and may be used for repeated sectioning over long periods of time (often several weeks or months).



TEXT-FIG. 3. TEM micrograph showing corrugations throughout the section, caused when the embedding medium is too soft, × 8000 (*Dictyonema rarum* Wiman, SM X1193).

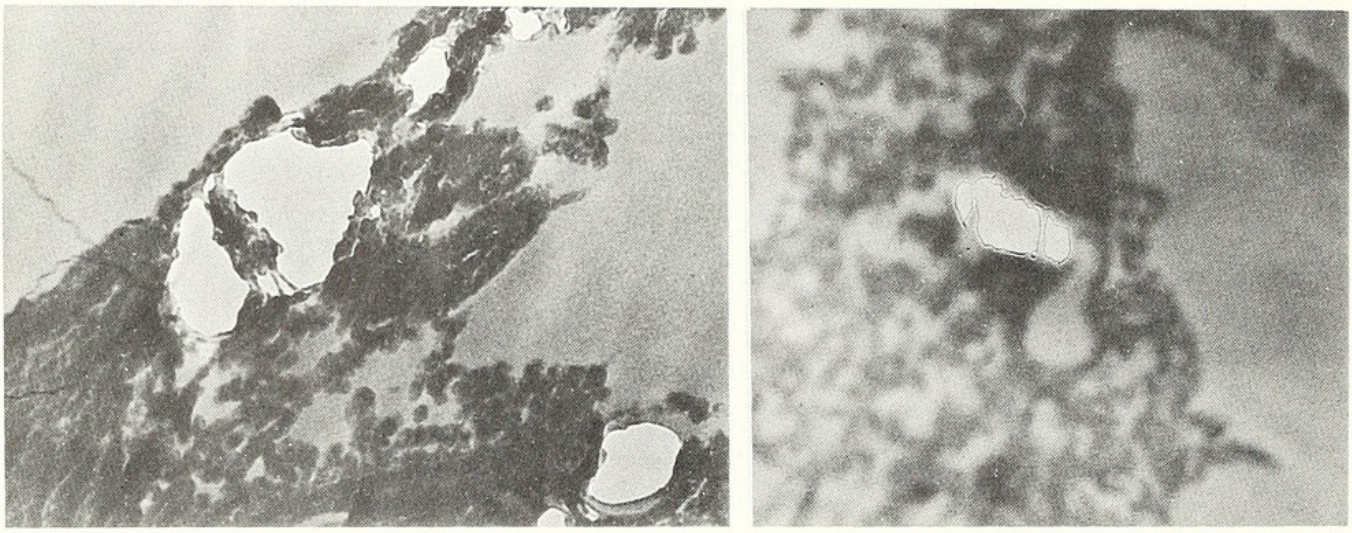
A picking brush dipped in chloroform and held over the surface of the water bath causes the sections to flatten, due to the heavy vapour. This eliminates, to a certain extent, deformation that might have occurred during sectioning. An uncoated copper grid is held with fine forceps and placed matt side down on top of the floating sections, which will then adhere to the grid. After drying matt side up on filter paper the grids are ready for use in the electron microscope. We have found that in general it is not necessary to coat prepared grids with carbon (but see also Crowther and Rickards 1977).

It is more informative to study several sections cut in serial order than single sections, since any contaminants introduced during sectioning can be recognized more easily when seen in successive sections. If the 'ribbon' of cut sections is transferred to the grid intact, it may be possible during TEM examination to obtain a three-dimensional understanding of that part of the graptolite ultrastructure.

PHOTOGRAPHY

Initially problems were encountered with lack of contrast in the electron micrographs, a not uncommon feature of electron microscopy; the thinner the section the less contrast there will be between specimen and background. Since thinner sections provide better resolution, it is necessary to

try to increase the contrast in some other way. One such method is to take photographs slightly underfocused. True focus on the electron microscope is found at the point of least contrast. This may be seen by observing fresnel fringes on the edges of the specimen or around the edges of adventitiously placed holes (text-fig. 4). (Fresnel fringes arise from the interference between scattered and unscattered electron beams; see Agar and Chescoe (1974) for a full discussion on their formation.) When the specimen is overfocused, the edge is outlined by a pale diffraction band with a concentric dark one (text-fig. 4B); when underfocused, a pale band (or fresnel fringe) follows the edge (text-fig. 4A). True focus occurs where the fresnel fringe disappears but contrast appears to be minimal. Thus it is often preferable to take photographs slightly out of focus in order to heighten contrast whilst increasing, of course, the risk of lower resolution. Contrast may also be increased at the film development stage. Micrographs are taken using 'Kodalith MP11 Ortho film 2577' on an 'AEI Corinth 500' electron microscope and are developed for three minutes at 13 °C in 'Ilford Phenisol' developer, diluted in the proportion 1:6.



A B

TEXT-FIG. 4. TEM micrographs showing the pattern produced by fresnel fringes. A, overfocused; B, underfocused. Both SM X1193, × 5000 approx.

The micrographs are then printed on 'Ilford Ilfoprint grade 4' paper using a 'Beseler MCX' enlarger. TEM micrographs should be taken as quickly as possible, since contamination builds up quite quickly on the specimen and may adversely affect both contrast and resolution, blurring the section. SEM photographic techniques have been discussed comprehensively by Crowther and Rickards (1977, pp. 11–12) and are not enlarged upon here, except to say that most of our SEM work is now carried out using a 'Philips 501B' electron microscope and 'Ilford FP4 (70 mm)' film processed according to manufacturer's instructions.

MUSEUM STORAGE

Storage of isolated reference material used under the light microscope is a relatively straightforward matter. The authors have been working on graptolites prepared and stored in glycerine, corked, and sealed with candlewax in 1895 and they are as satisfactory today as presumably they ever were. They respond well to both TEM and SEM studies, and the only deterioration evident is of the glycerine, not the graptolite. In some tubes the glycerine has gone slightly brown, though it is still transparent. At the Sedgwick Museum, graptolites have been similarly stored in glycerine for twenty years.

However, in order to facilitate normal examination by research workers, they are stored in the type of plastic container illustrated (text-fig. 1) in which very viscous glycerine only 5–10 mm deep is used, which greatly lessens the chance of spillage. Yet the container is not sealed, has a sliding lid, and the specimens can be easily examined under the light microscope, particularly as there is no problem with the sphericity of the container. There is no reason to suppose that material, so stored, will not last fifty years and normal curatorial procedures can be adopted by the museum staff.

SEM stubs pose a more difficult problem. The Sedgwick Museum has fifteen-year-old mountings which have been re-used successfully, but deterioration is apparent, especially in the glues or gums used to mount the specimens on the stubs but also in the coating used. The question of glues has been examined very thoroughly by Dr Jenny Chapman (*in prep.* and *pers. comm.*), and it seems unlikely that even well-mounted graptolites will last twenty-five years. Therefore, whilst normal curatorial procedures can be applied to stubs, and are at the Sedgwick Museum, the following items take on greater importance in curation: 1, the photographic negative (and prints); 2, unmounted topotypes (preserved in glycerine); and 3, topotype rock samples known to have yielded the originals.

The most difficult preservational problem concerns TEM ultrathin sections mounted on grids. Although Sedgwick Museum specimens have been re-used successfully after a period of five years, it is unlikely that grids will survive a decade; re-use itself usually causes rapid deterioration so that further use is unlikely. Therefore, the film negatives are vital from a curatorial and research standpoint and there seems no good reason why, properly stored, they should not last for a century. Thus the film negative becomes the 'specimen' for all future research reference. A supplementary part of the specimen is that remaining in the unsectioned stub. Although this has not appeared on film, it is a potential source of at least partial confirmatory work on the same specimen and hence should be catalogued as a very important part of the original. The life of a specimen mounted in resin depends upon the life of the resin, which may deteriorate in, at most, a few years, making further sectioning difficult (at least with a valuable diamond knife), but the mounted specimens may last for several decades as far as light microscopy is concerned.

It is clear from the authors' work that curation must be built into the procedural system and planned for at an early stage of the work. Much the best system is to curate all parts and products immediately after they have been produced, any delay merely contributing to the possibility of very small items being separated from each other and being effectively lost.

Acknowledgements. We gratefully thank Roger Northfield (Department of Zoology, University of Cambridge) for all his help with ultramicrotomy, and David Bursill for his constant assistance during experimental photographic work.

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Typescript received 20 October 1984
Revised typescript received 29 January 1985