II. THE SPECIMEN

II.A. BIOLOGICAL SPECIMEN PREPARATION TECHNIQUES

Biological specimens can be prepared in numerous ways for examination in the TEM. Methodology has reached a stage where reproducibility of specimen preparation is routinely possible in many instances. A **basic aim** is to obtain morphological information and obtain it by reproducible methods that can be repeated anywhere in the world, so the results obtained by one laboratory are strictly comparable with those obtained by another. Reproducibility strengthens the belief that micrographs provide faithful reflections of the native state of the specimen. The risk of misinterpretation from technique-induced artifacts is lower as preparation techniques become more refined. It often helps to study a given sample with a variety of preparation techniques, as each can provide independent and self-consistent information.

Due to time limitations, only a selected set of the more commonly used specimen preparative techniques is discussed in this class. These include:

1. TEM Grids and Support films
2. Thin-Sectioning
3. Negative Staining (Positive Staining)
4. Metal Shadowing
5. Freeze Drying/Fracturing/Etching
6. Unstained, Vitrified Specimens

II.A.1. TEM Grids and Support Films

Whether the specimen is a tissue section or a suspension of particles, it must be supported in some way to position it on the optic axis of the microscope. Most often, specimens are mounted on a thin, electron transparent film. Twenty to fifty nanometer thick layers of evaporated carbon are almost universally used for this purpose since they:

- Are relatively transparent
- Have limited granularity
- Are relatively stable to irradiation

a. The TEM grid

The specimen and supporting film require the mechanical support of a metal grid, which is necessarily electron opaque. The number of grid bars and percent open area can vary quite widely with different types of grids (Fig. II.1). 200-400 mesh (lines/in) grids are commonly used. Most EM grids are made of copper because this metal is non-ferromagnetic and thus minimally distorts the magnetic field of the objective lens. Even so, it is usual practice in high-resolution studies to avoid, if possible, recording images of specimens that lie close to the grid bars.

Modern grids are generally made by a photographic electro-deposition process, which makes it easy to produce cheap, disposable grids with a wide variety of meshwork patterns. The copper mesh also rapidly conducts heat away from the support film and helps prevent thermal expansion and hence movement of the specimen under electron irradiation. Grids may be used bare or, more commonly, covered with a thin film depending on the nature of the specimen being studied (Fig. II.2). Thin sectioned specimens, for example, are sometimes examined on bare grids, but for highest resolution work, the sections must be mounted on filmed grids or, better yet, on net films (see § II.A.1.f).

b. Plastic or carbon support films

TEM grids have openings small enough to allow an extremely thin (20 nm or less) film of a suitable electron-transparent substance (plastic or evaporated carbon) to be stretched across them without breaking when gently handled or when irradiated with the electron beam. Because the support film is often as thick or thicker than the specimen itself, contrast introduced by the support may be comparable to that of the specimen. Aperture (or scattering or 'amplitude') contrast in the support film is generally negligible when thin support films such as 20-50 nm carbon are used. At small to

moderate levels of defocus (200-500 nm underfocus) phase contrast produces a granularity in the support film image that is superimposed on the specimen image and may lead to incorrect interpretation of specimen features. This is due to the formation of a phase contrast image of the randomly arranged carbon atoms in the film (with interatomic distances of about 0.27 nm).

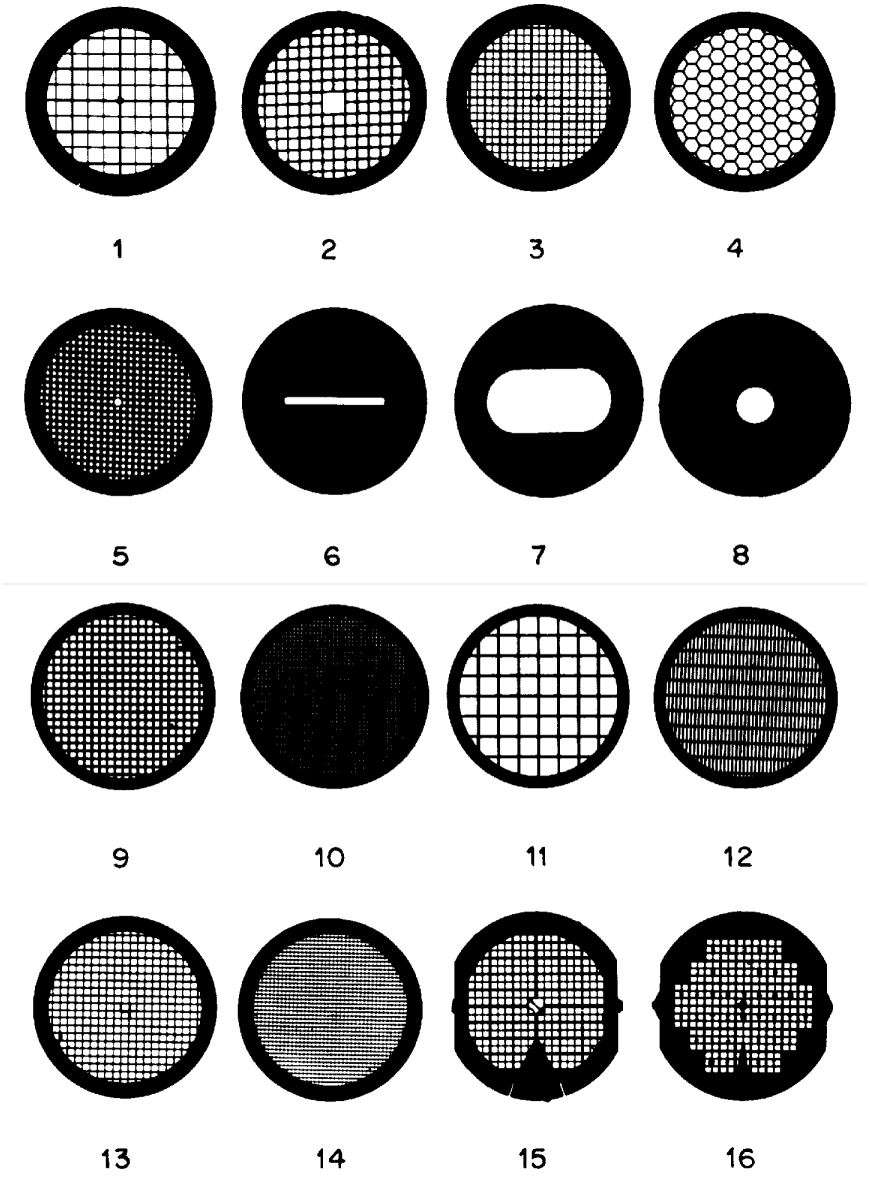


Fig. II.1. A representative selection of commonly used 3 mm grids. (From Meek, 1970, p.322)

Films are generally one of three types:

- Plain plastic such as **collodion** or **Formvar**
- Plastic that is coated (stabilized) with evaporated carbon
- Plain carbon

Plain carbon films are generally the choice for highest resolution work because:

- They are tough and withstand the electron beam.
- They conduct electricity and hence reduce specimen drift due to charge effects.
- They conduct heat from the specimen to the grid bars, thus reducing thermally induced drift.

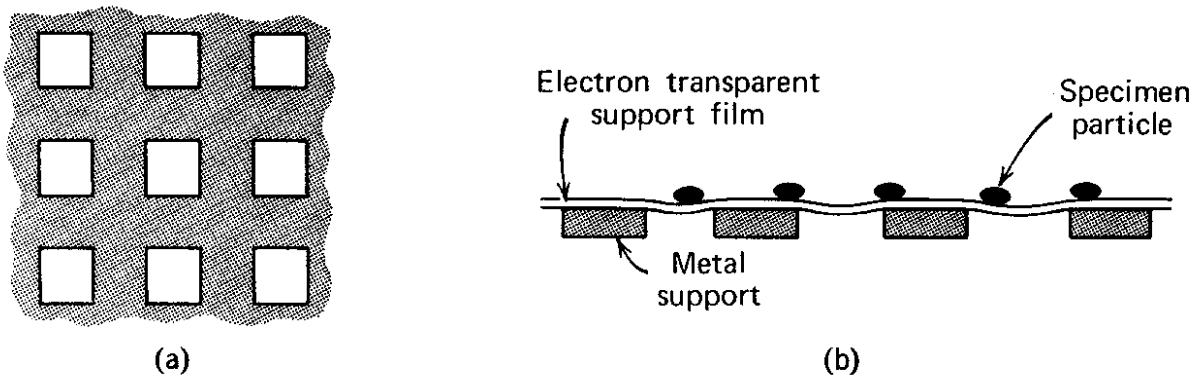


Fig. II.2. Grid for electron microscope specimen. (From Slayter, 1970, p.391)

c. Preparation of plastic and/or carbon films

There are many recipes for preparing good support films. Each EM user generally masters two or three suitable procedures. The nature of the specimen to be examined determines to some extent what type of support film is best to prepare. Plastic films are easier to make than pure carbon films. The most common plastics used are collodion and polyvinyl formal (trade name **Formvar**). Carbon films are preferred for use in studies where resolution is a critical factor.

Plastic films are generally made from a dilute (<0.5%) solution of the plastic in a non-polar solvent. For example, ethylene dichloride or chloroform is used with Formvar, and amyl acetate is used with collodion. A thin (<20 nm) film of the plastic is then prepared by one of two standard techniques:

- Dip a cleaned glass microscope slide into the solution, let it drain and dry. Then float the plastic film from the surface of the slide by slowly dipping it into a vessel of water (Fig. II.3).
- Place a drop of the solution on the surface of water and allow it to spread out and dry. This leaves a thin layer of plastic on the water surface. This method tends to produce thicker and more uneven films compared to the first method (not illustrated).

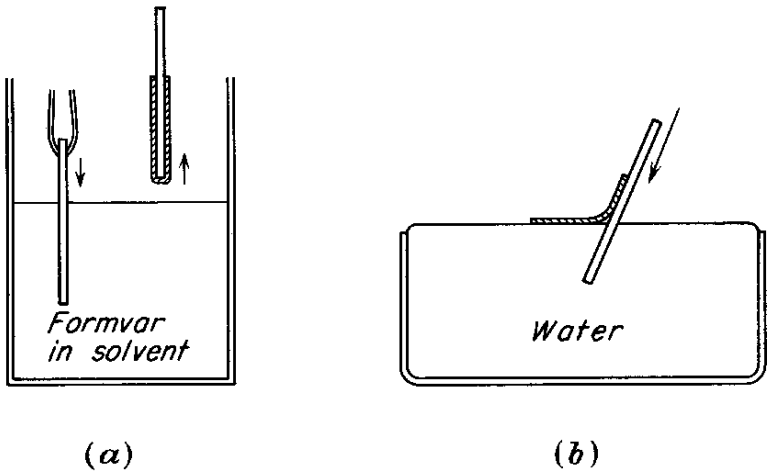


Fig. II.3. Stripped films. (From Hall, 1966, p.286)

The film floating on the surface of the water should appear an even grey to dark grey color when viewed by reflected light. If it appears yellowish or uneven, it is too thick and unsuitable. The film is transferred to grids by laying the EM grids shiny-side up (dull side down) onto the plastic film. The film and grids are picked up with a piece of hard filter paper or with Parafilm or with a glass slide and then set aside in a dust free place to dry (Figs. II.4, II.5). Alternatively, the grids can be submerged beneath the plastic and lifted up through the film (Fig. II.6). Grids can then be used as is or they may be coated with an evaporated layer of carbon.

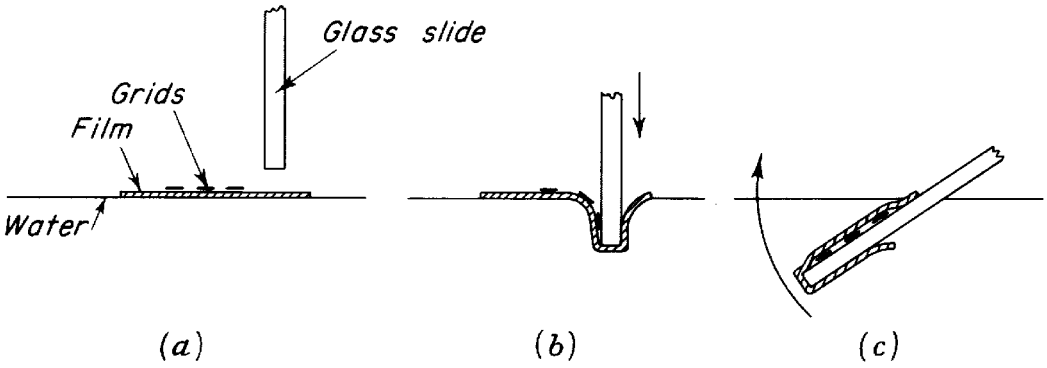


Fig. II.4. One method of preparing filmed grids. (From Hall, 1966, p.284)

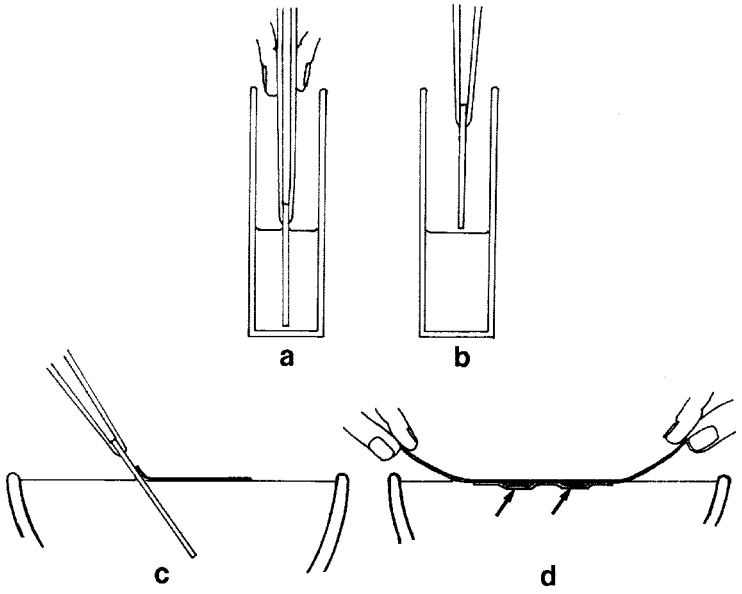


Fig. II.5 The preparation of Formvar specimen support films: (a) A cleaned glass slide is dipped in the solution of Formvar in chloroform; (b) The slide is withdrawn and drained in the presence of chloroform vapor; (c) The thin plastic film is floated onto a water surface; (d) After placing specimen grids (arrows) on the film, it is removed from the water using a strip of paper. (From Willison and Rowe, 1980, p.64)

Carbon-Formvar films can be easily converted into carbon-only films by dissolving away the plastic. The carbon-plastic films are placed plastic-side down onto a piece of filter paper soaked in a solvent, which dissolves the plastic. After a few hours, the grids are moved to a dry piece of filter paper and allowed to air dry.

Another method for preparing pure-carbon films is to evaporate a layer of carbon directly onto the surface of a freshly cleaved piece of mica (Mica is a group of minerals characterized by perfect cleavage into thin flakes in one direction and by the molecularly flat, clean surface that is produced in each flake). The carbon is then floated off onto water and transferred to EM grids in one of two ways:

- TEM grids can be brought up through the water from below the film or,
- The film may be carefully lowered onto grids, situated beneath the water surface on a piece of wire mesh, by slowing removing the water from the vessel.

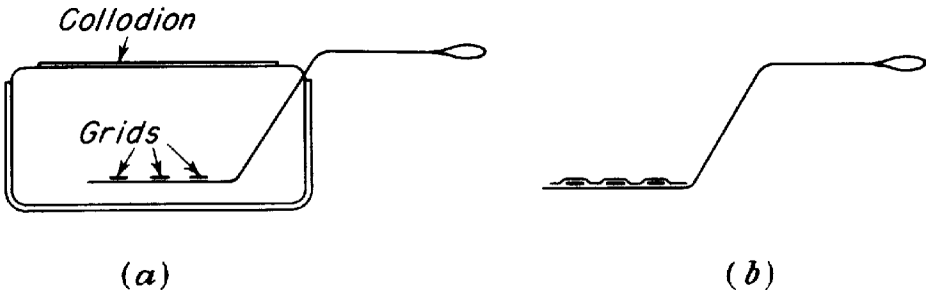


Fig. II.6. Preparation of filmed grids. (From Hall, 1966, p.284)

d. Carbon evaporation

The apparatus used to evaporate thin layers of carbon consists of a bell jar that is evacuated by a diffusion pump and backed by a mechanical pump (Figs. II.7, II.8). Inside the vacuum chamber is a base plate with vacuum and electrical feed-throughs. The mechanical rotary and oil diffusion pumps are used in series to produce a vacuum of about 10^{-5} Torr, which is low enough so the mean free path of vaporized atoms is long compared with the distance they are to travel. A current of 50 amps is passed through two rods, one sharpened down to 1 mm diameter and the other 3 mm, to evaporate the carbon (Figs. II.9-II.11). The rods are held in contact by a spring while the current flows through the rods. Heat produced at the junction is sufficient to cause rapid evaporation. The amount evaporated can be estimated by the decrease in length of the narrow portion of the carbon rod or by noting the blackening of a piece of filter paper in the region surrounding the shadow cast by a small object (*e.g.* thumb tack) placed on the paper.

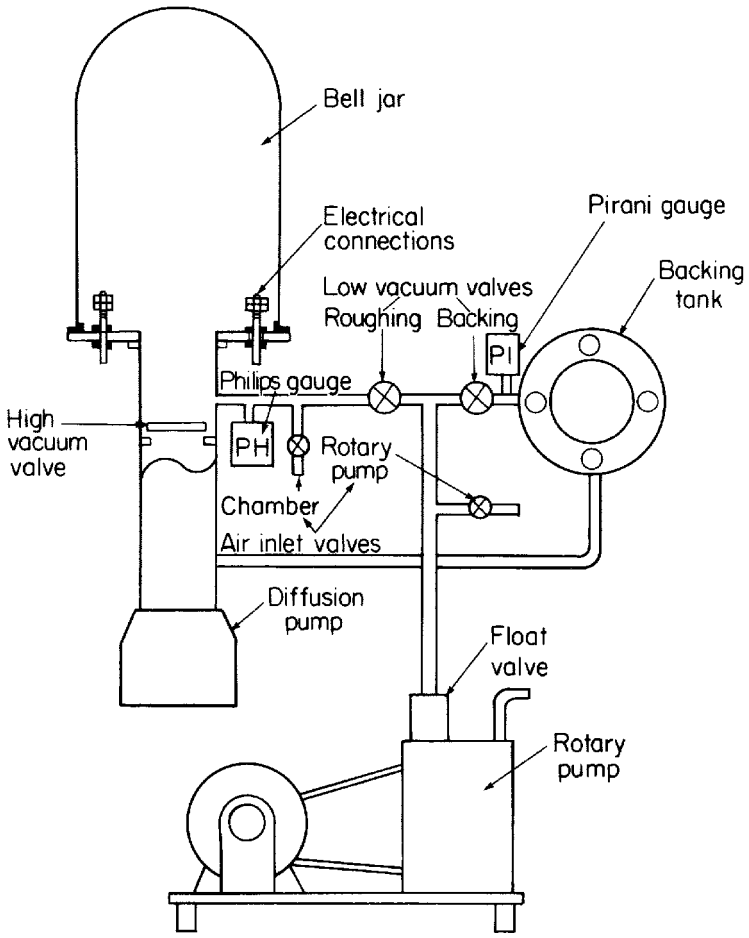


Fig. II.7. The vacuum system of an Edwards 12E1 evaporator. The backing tank can be used for desiccating photographic material for the electron microscope. (From Meek, 1970, p.473)

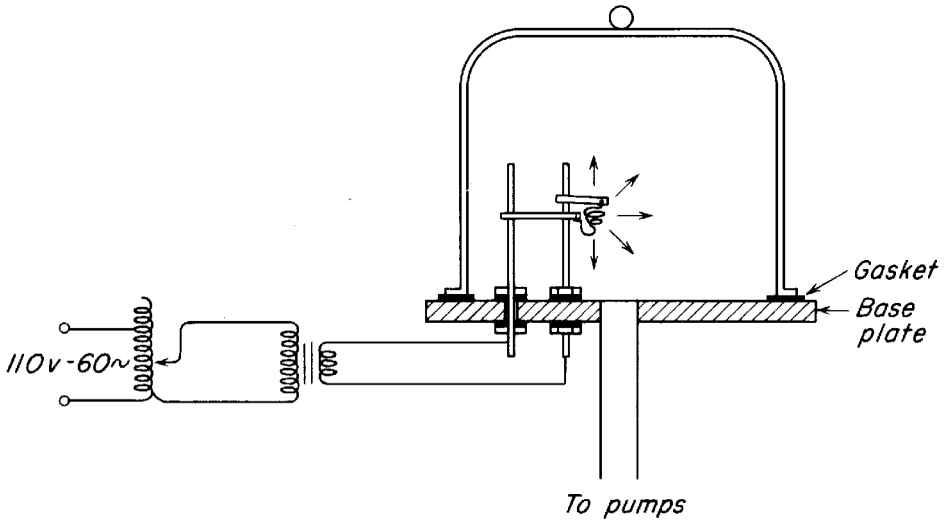


Fig. II.8. Apparatus for evaporation of substances in a vacuum. (From Hall, 1966, p.279)

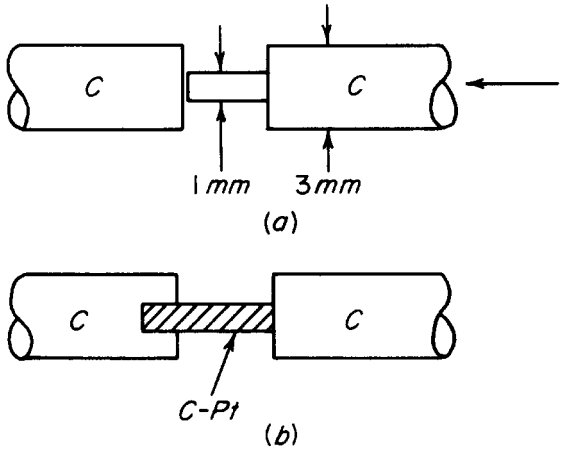


Fig. II.9. Carbon rods for the evaporation of (a) C and (b) C-Pt. (From Hall, 1966, p.282)

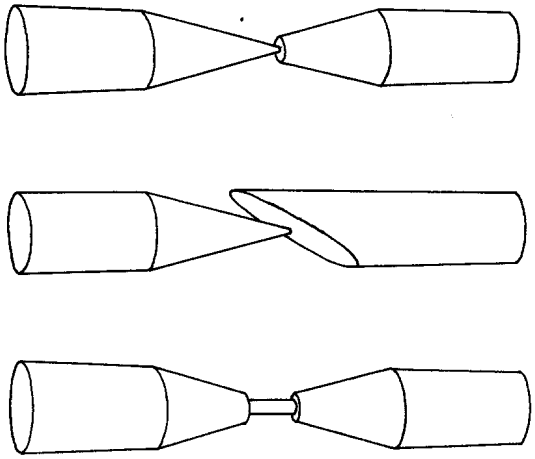


Fig. II.10. Three possible arrangements of the two graphite rods constituting the carbon 'arc'. (From Willison and Rowe, 1980, p.34)

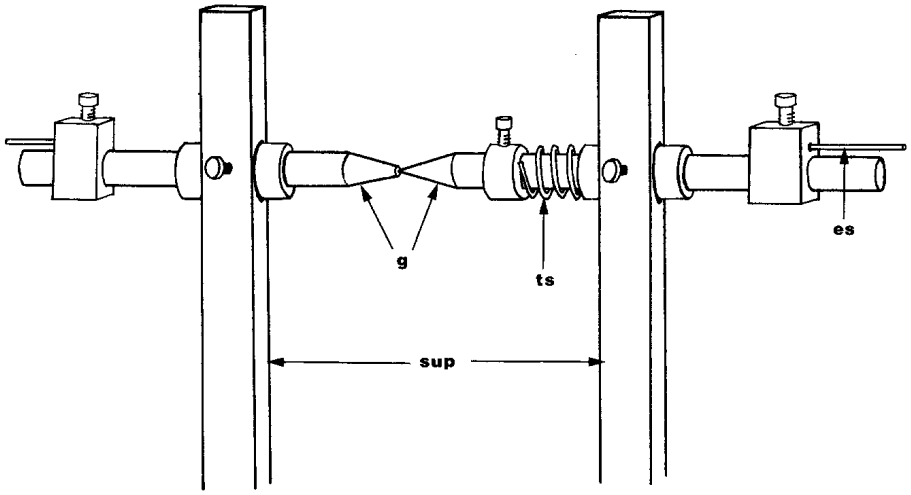


Fig. II.11. A typical graphite 'arc' used in carbon evaporation. Electrical supply (es); tensioning spring (ts); sharpened ends of the two graphite rods constituting the 'arc' (g)' supports for the 'arc' within the coating unit (sup). (From Willison and Rowe, 1980, p.34)

e. Preparation of holey films

Carbon-Formvar films pierced with a large number of circular holes with sharp edges and diameters in the range 0.1-1.0 μm make excellent test objects for astigmatism corrections and resolution checks (Fig. II.12a,b). A variety of methods are used to prepare holey films. Unfortunately, there aren't any "fool-proof" methods as seems to be true for preparing regular films. There are two basic protocols for producing the holes:

- Minute droplets of water are condensed onto the surface of a drying Formvar film. As the film dries, the water droplets pierce the film leaving circular holes.
- Formvar is dissolved in a solvent that contains water. This mixture, including a non-polar solvent for the Formvar (*e.g.* ethylene dichloride) plus a partially polar solvent miscible with both water and Formvar (*e.g.* glycerol) will dry such that the more volatile solvents evaporate first, leaving minute water droplets to pierce the film.

The TEM user should be able to distinguish true holes from pseudo-holes, which, at low magnification, appear to be suitable, but, on closer inspection at high magnification, are found to have a thin film of Formvar over them. Pseudo-holes can sometimes be removed by dipping the filmed grids in acetone for a second or two, or, for better control, the filmed grid is held over acetone vapors for a few seconds.

The holey Formvar film is detached from the microscope slide onto a water surface, the TEM grids are applied, picked up, dried, and a layer of evaporated carbon is added in the same manner as is performed with regular films.

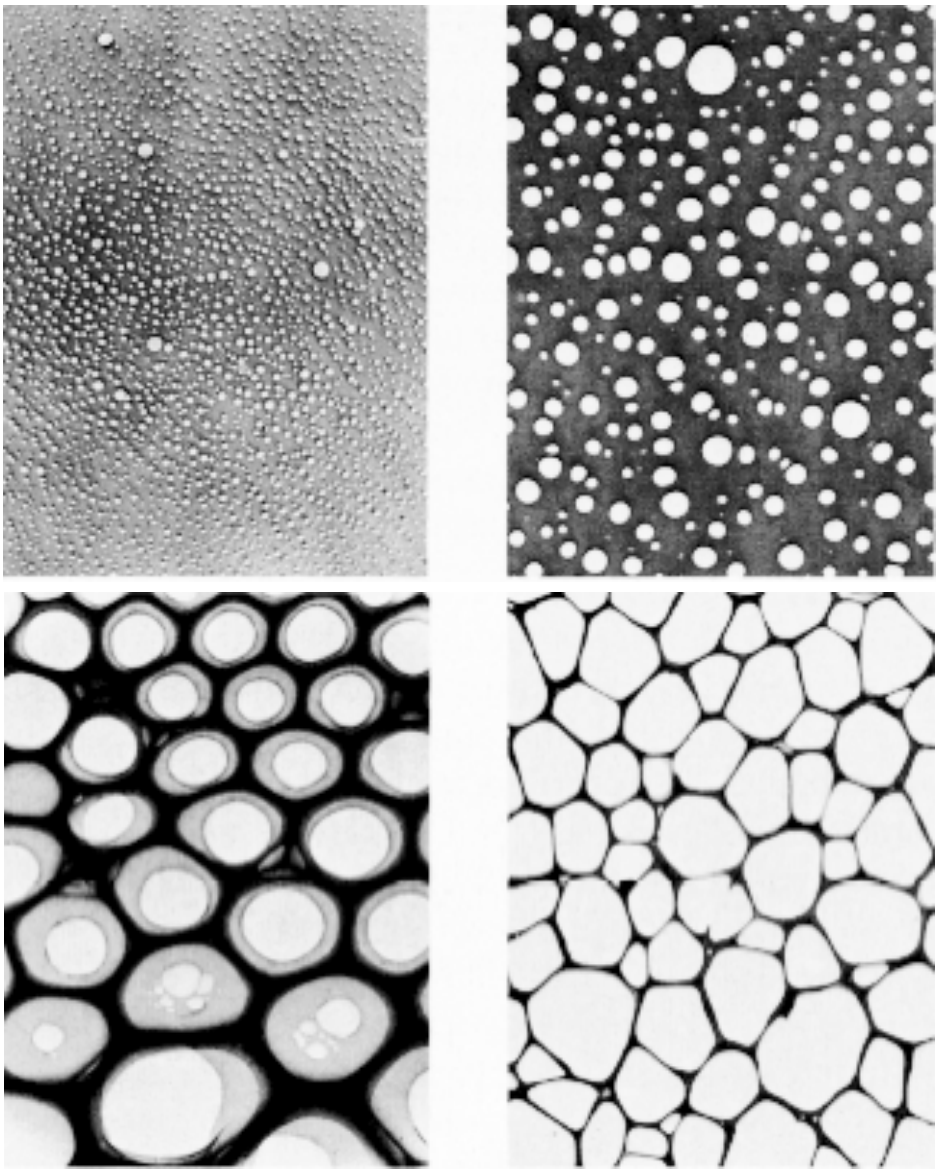


Fig. II.12. The 'holey carbon film' test object. (Top left) is a low-power micrograph of a well-prepared film with numerous holes in the right size range (~1,500X); (top right) is an enlarged view (~5,400X), showing almost circular holes with clearly demarcated edges between 1.0 and 0.1 μm in diameter. The thickness of the film is even, right up to the hole edges; (bottom left) is an example of a very poor film with partly perforated holes of too large a diameter, with a few ragged small holes in the septa; (bottom right) is the result of carrying the last panel to the extreme - a carbon-Formvar 'net', which is some-times used as a support for extremely thin epoxy resin sections when the very highest resolution is required from them. (From Meek, 1970, p.325)

f. Formvar nets

These are distinguished from holey films by the size range of the holes formed (usually >1 μm in diameter). A "net" by definition generally has more area occupied by "hole" than by film (Fig. II.12d). This type of film is excellent for studying extremely thin sections where areas of the section can be photographed at the highest possible resolution through holes in the net. Nets are prepared in essentially the same way as holey films. The simplest procedure is to hold a drying Formvar slide over the surface of boiling water for about one second. A fairly thick (50 nm) layer of carbon is generally evaporated onto net films to give them added stability for supporting sectioned material.

g. Commercially manufactured holey films

The rising popularity of electron cryo-microscopy (see §II.A.6) and automated TEM methods, which routinely rely on the use of holey carbon support films, has stimulated a sizeable demand for grids manufactured with holes of defined size and distribution. At least two companies, Quantifoil (www.quantifoil.com) and Prochips (www.protochips.com), offer a variety of carbon-coated grids that are widely used in cryo-microscopy studies (Fig. II.13). Both QUANTIFOIL® and C-Flat are perforated carbon support foils with pre-defined hole size, shape and arrangement and are offered with circular and square, orthogonal arranged holes. Films with different hole sizes and bar widths are available. For example, QUANTIFOIL® R2/2 grids come with circular holes that are 2 μm apart and 2 μm in diameter. The hole size chosen depends on the magnification used (typically between about 30,000X and 50,000X), and on whether or not one wishes to include support film in the image.

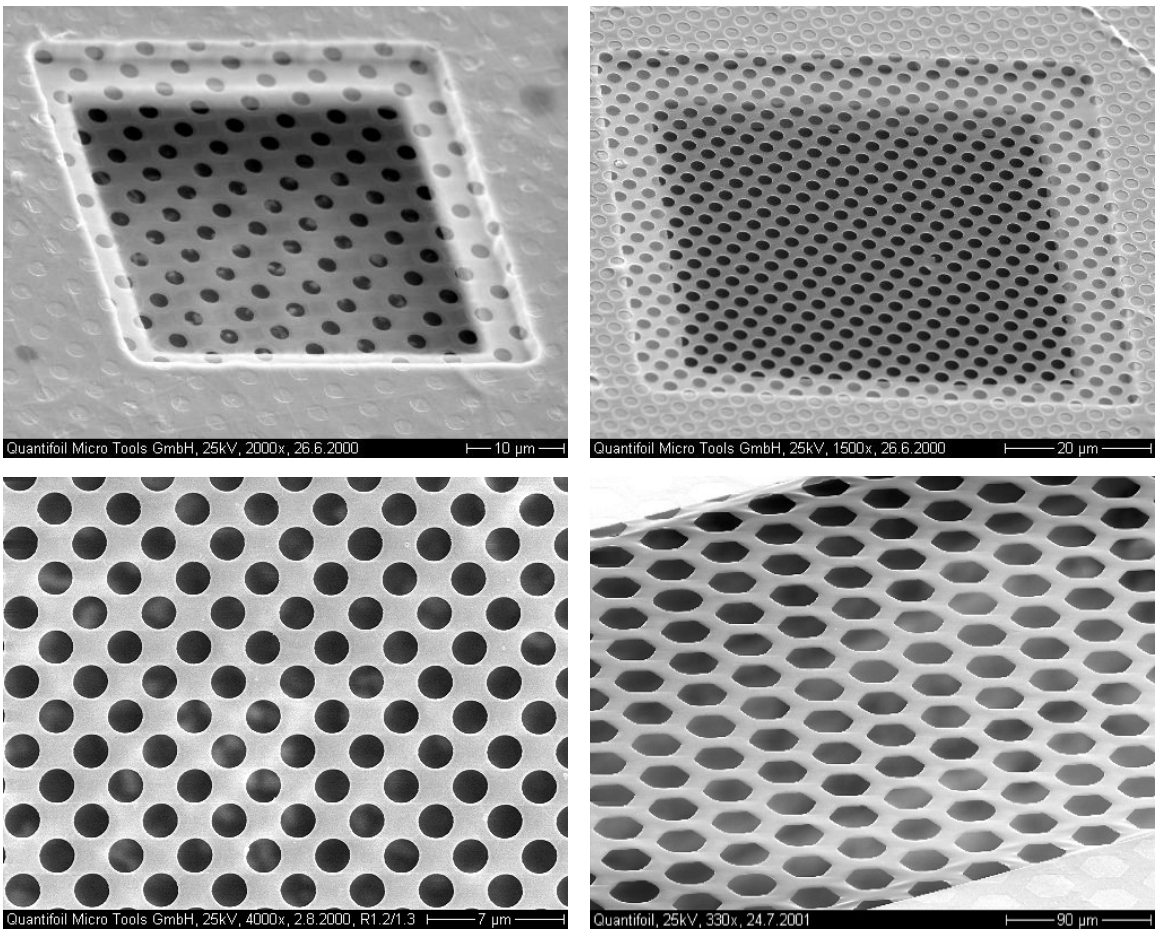


Fig. II.13 Four examples of Quantifoil holey grids. The top row shows Quantifoil R 2/2 and R 2/1 grids and the bottom left shows the Quantifoil R 1.2/1.3 grid. The first and second numbers specify in μm units the diameter of the hole and the space between holes. Hence, the center-to-center spacings between the holes in these three grids are 4.0, 3.0, and 2.5 μm, respectively. The last grid (lower right) is the Quantifoil HEX15, with hexagonal shaped holes.

II.A.2. Thin Sectioning

Approximately 50% of a 50 kV beam of electrons can penetrate ~100 nm of a specimen with a density of 1 g/cm^3 (the approximate density of typical biological specimens). Thus, it is not possible to study whole cells in a conventional 20-100 kV TEM. It is necessary to obtain specimen samples that are thinner than ~90nm and ideally 50 nm thick or even thinner. Before this is done the sample must be stiffened so it is capable of bearing up against a sharp cutting edge sufficiently well, such that very thin slices can be cut. Before this the specimen must be "killed" (fixed) in such a way as to preserve faithfully every detail down to the molecular architecture just as it was in life. A critical factor in obtaining good experimental results is the isolation of the tissue in as close as possible to *in vivo* conditions. The term ***tissue*** is used in a liberal sense to include specimen samples whether they are from animal, plant, bacteria, algae, or other organisms and living matter.

Most specimens can be excised from living tissue and gently cut into small pieces within a few minutes, thus minimizing trauma and the time that the tissue is deprived of its essential nutrients. Slicing of excised tissue is best done on a non-absorbent surface (*e.g.* polyethylene slab) in a droplet of fixative. The tissue should generally be cut into pieces with at least one dimension smaller than 1 mm to assure rapid fixation of the entire piece of tissue.

Tissue can also be fixed *in situ*, for example by perfusing into living, anesthetized animals. This can greatly minimize the effects of cutting on unfixed tissues and of anoxia resulting from cutting off circulation.

Specimens such as protozoa, cell suspensions, algae, and bacteria can be prepared by being placed directly into fixative. If they are very small, the specimens can be centrifuged to form a pellet for easier handling immediately after the fixation. The speed of centrifugation necessary to form a pellet is dependent on the nature of the specimen.

a. Fixation

The main goal of fixation is to maintain the original form of the specimen so it can resist the effects of subsequent steps in the preparative procedure. The aim is to preserve every detail of cellular ultrastructure right down to the molecular level exactly as it was in life the instant before cell death. The first problem is to obtain cells in their normal living condition, thus most tissues have to be obtained from a living organism. A variety of methods have been devised for getting fixative solution to the desired tissue. Because the time of penetration of the fixative into the tissue is critical, the tissue must generally be cut into very small pieces, preferably cubes no larger than 1mm^3.

1) Fixatives

The primary purpose of the fixative is to solidify the protein sol bounded by the cellular membranes and to preserve the spatial relationships of the various organelles as they were at the instant of fixation. It should also render insoluble all the other chemical constituents of the cell such as nucleic acids, nucleoproteins, carbohydrates and lipids. Some fixatives (such as osmium tetroxide) also provide electron contrast.

Osmium tetroxide (OsO_4)

The detailed nature of the interactions of OsO_4 with cellular components is not fully understood although it is known to react with unsaturated lipids and with tryptophan and histidine in proteins, thus cross-linking polypeptide chains together. OsO_4 acts to stabilize cellular proteins that form the matrix of the cytoplasm. A number of factors, including pH, temperature and time of fixation are critical for good fixation. For example, the time of fixation is normally 30 to 90 minutes. If fixed for too short a period, there may be inadequate stabilization of the specimen, whereas, fixation for prolonged periods can occasionally lead to solubilization of some tissue components. OsO_4 is usually prepared in a buffered solution at near neutral pH. It must be noted that various buffers produce different appearances in ultrathin sections of tissue fixed with OsO_4 at identical concentration and pH. For example, differences in the preservation of nuclear chromatin, endoplasmic reticulum, mitochondria, and background cytoplasm have been observed.

Osmium is good for rendering lipid insoluble but has no effect on carbohydrates such as glycogen. It stabilizes protein sols by combining chemically with them and forming cross-links, not by precipitating them. It preserves lipids by forming addition compounds with unsaturated fatty acid chains. It is good at preserving the phospholipoprotein membrane skeleton of the cell. It preserves nucleic acids and carbohydrates poorly, but nucleoproteins well. Since it combines chemically with practically all the constituents of the cell, osmium metal remains behind in the fixed cell, attached firmly to the structures it stabilizes, and delineating them almost perfectly and increases the mass thickness of the specimen. It penetrates tissue very slowly due to the low diffusion rate of its large molecules.

Aldehydes

Formaldehyde and **glutaraldehyde** are the most popular aldehydes used as fixatives. They preserve structure excellently but allow lipids to be completely extracted by the alcohol used as the dehydrating agent (§ II.A.2.b). They preserve glycogen well but provide no additional electron contrast. They penetrate tissue rapidly and have the property of stabilizing glycogen so its subsequent loss with OsO_4 is prevented. They preserve structures such as microtubules, which are poorly or not at all preserved by OsO_4 and they stabilize nucleoproteins better than OsO_4.

Glutaraldehyde is often used in conjunction with OsO_4 to give better specimen stability during subsequent dehydration, embedding, and electron microscopy. Two major advantages of aldehyde fixatives over OsO_4 include:

- More rapid penetration of tissues resulting in less distortion and better preservation of structural relationships
- Lower tendency to extract cytoplasmic components resulting in improved fidelity of ultrastructure.

Permanganate

This is a strong oxidizing agent that preserves cell membrane structures and is particularly good in preserving myelin. It also acts as an electron stain by precipitating coarsely granular, electron dense material on the membranes. DNA and RNA-containing elements are not satisfactorily preserved, however. Thus, permanganate is better used as a selective rather than a general-purpose fixative. The rate of penetration of permanganate is, like OsO_4, slow in comparison with that of the aldehydes. The selective action of permanganate serves to make membranes stand out because of its strong reaction with the phospholipid component of the membrane.

No one substance is likely to provide all the functions required by an ideal fixative. Most modern work is therefore performed with material that has been fixed first in an aldehyde and then fixed in OsO_4.

2) Buffers and additives

Effects due to osmotic pressure differences between the fixative solution and the cytoplasmic sol may cause disruptive effects (*i.e.* shrinkage and swelling) to the organelles. Thus, neutral salts or other inert substances are used in the fixative solution to make it isotonic with the tissue fluid and a buffer is used to maintain the pH of the fixative solution at the physiological value in spite of the chemical activity of the fixative.

A commonly used buffer contains two components: sodium acetate to provide buffering capacity in the acid range and sodium barbiturate (Veronal or barbitone sodium) for the alkaline range. The necessity for using additives (*e.g.* sodium chloride or sucrose) to increase osmotic pressure is a controversial matter, some saying the additives are essential and others claiming they are useless.

3) Fixation Technique

A two-stage fixation procedure using 1-6% glutaraldehyde buffered with phosphate (0.05-0.1M, pH 6.8-7.6) followed by OsO_4 is now almost universally used. Primary fixation may be performed (usually on ice) for 1-3 hours or more depending on the size and permeability of the cut tissue samples. After primary fixation, the unreacted glutaraldehyde is washed out since it combines with

and reduces the OsO_4, causing unwanted precipitation in the tissue.

Since OsO_4 penetrates very slowly into **fixed** tissue, the blocks (chunks of tissue) must be made as small as possible before the secondary fixation (<0.5mm in the smallest dimension). The secondary fixation is normally carried out at room temperature with a 2% solution of OsO_4 (buffered or unbuffered).

Fixation steps are generally performed in a fume hood, especially when OsO_4 is used since it is extremely volatile and toxic (instantly kills any epithelial cells it contacts).

4) Other fixation methods

The most commonly used physical (*i.e.* non-chemical) method of fixation is that of freeze-substitution in which small pieces of the specimen are rapidly frozen by plunging them into isopentane cooled to liquid nitrogen temperature. To prevent ice crystal formation, the tissue is first soaked in cryo-protective agents such as glycerol, ethylene glycol, or dimethyl sulfoxide and is then rapidly frozen. Water is then removed by sublimation, with the specimen kept at about -20°C and under vacuum. A plastic monomer (under vacuum) is infiltrated into the frozen-dried tissue to embed it, and the polymer is then polymerized. This technique is especially good for preserving enzymatic activity in the tissue sample. Thus, histochemical reactions can be performed on the sections to localize the sites of activity of certain enzymes.

b. Dehydration

The goal of the dehydration step is to replace all the free water in the specimen with a fluid that is miscible both with water and with the embedding resin. Agents such as ethyl alcohol, methyl alcohol, isopropyl alcohol, acetone, or the monomer of a water-soluble plastic embedding medium are used to dehydrate the specimen. Ethyl alcohol is the most widely used dehydration agent because it does not harden the tissue and make it too brittle for subsequent ultrathin sectioning. Other agents can be used depending on the nature of the embedding material being used. For example, acetone is used when the polyester resin Vestopal is used. Inert compounds such as ethylene and propylene glycol can be used as effective dehydrating agents because they serve both to displace tissue water and stabilize the cell's macromolecular systems. Hence, these inert compounds allow most cytoplasmic proteins and the fine structural relationships between them to be retained.

The duration of the dehydration step is normally kept short to prevent extraction of tissue components and subsequent shrinkage. The specimen sample is transferred into mixtures of water and dehydrating fluid of decreasing water concentration. The usual routine calls for rapid dehydration: short treatment with 70% alcohol, then transfer to 95% and finally to 100% for about an hour. After the specimen has been transferred to 100% dehydrating agent, it may then be transferred to a fluid completely miscible with both alcohol and the resin monomer. Such a fluid is propylene oxide (1,2-epoxypropane: EPP), which is completely miscible with the epoxy resins typically used for general embedding purposes.

The processes of dehydration and infiltration should be carried out as rapidly as possible because all the reagents used are powerful lipid solvents, and remove a significant amount of lipid even after it has been fixed with OsO_4.

c. Block staining

Further differential electron contrast can be added after osmium fixation and before the tissue is embedded. The washing and dehydrating fluids can be used to add further stains such as uranyl acetate, phosphotungstic acid, or potassium permanganate.

d. Embedding

In the early days of thin sectioning, paraffin waxes were used as embedding media but were found to be too soft to enable sections thinner than about 1 μm to be cut. The first embedding medium found suitable for electron microscopy was poly-butyl methacrylate. The majority of sectioning studies presently employ one of the epoxy resins.

1) Properties of an ideal embedding medium:

- Soluble in ethanol (or acetone) before polymerization
- Does not itself chemically modify the specimen
- Does not physically disrupt or distort the specimen
- Hardens uniformly
- Produces a block hard enough yet plastic enough to cut ultrathin sections
- Stable under electron irradiation

No one embedding medium possesses all of these properties.

2) Types of embedding media

Methacrylate

This penetrates tissues rapidly and is easily cut into sections in the thickness range 50-100 nm. Proper hardness of the tissue blocks is achieved by varying the ratio of a mixture of methyl methacrylate and n-butyl methacrylate monomers. Hardening of the blocks is the result of polymerization of the monomer molecules to form simple linear polymer chains.

Some problems encountered with methacrylate sections include:

- Shrinkage - up to a 20% decrease in volume is encountered when methacrylate polymerizes
- Polymerization damage causes specimen artifacts
- Instability of the sections under electron irradiation causing up to 50% sublimation of the methacrylate and subsequent distortion of the specimen.

The disadvantages of the methacrylates arise mainly from the fact that they form linear polymers that do not cross-link to form a stable, three-dimensional resin matrix.

Gross tissue artifacts are caused due to shrinkage of the methacrylate when it polymerizes. Cell components are dragged apart giving a vacuolated appearance to the embedded tissue. Evaporation of the resin in the electron beam leads to collapse of the unsupported structures on top of one another causing gross distortion of the ultrastructure. The evaporated plastic also deposits on the polepieces of the objective lens leading to astigmatism that becomes uncorrectable. Evaporation of the methacrylate does, however, result in an increase in specimen contrast. Evaporation can be reduced by pre-irradiating the section at low beam intensity and by sandwiching the section between protective films of carbon.

Water Soluble Embedding Media

Aqueous solutions of these media are used immediately after fixation, both as dehydrating agents and then, in pure form as infiltration and embedding agents. Three water-soluble agents include:

Glycol methacrylate (2-hydroxyethyl methacrylate) - sections tend to stretch in the electron beam.

Aquon - Water-soluble component of the widely used epoxy resin, Epon 812. It is of relatively low viscosity and sections fairly well.

Durcupan - water-soluble epoxy resin, which, when used without other added epoxies, gives very soft blocks. When sufficient additives are provided to improve its sectioning properties, most of the advantages associated with its water solubility appear to be lost.

Polyester Resins

Vestopal W, an acrylic resin, uses an initiator and activator. If alcohol (not miscible with Vestopal) is used to dehydrate the specimen, then the alcohol must be replaced with styrene, which is miscible with both alcohol and Vestopal. Alternatively, acetone can be used to dehydrate the specimen. Vestopal-W has the disadvantages of being very viscous and immiscible with ethyl alcohol, but the sections are very stable in the electron beam.

Epoxy Resins

These are a family of thermosetting synthetic resins, which, when mixed with suitable curing agents and heated, polymerize irreversibly into cross-linked, yellow-brown solids. The resin has two types of chemically reactive groups: epoxide end groups and hydroxyl groups spaced along the length of the chain. If a mixture of resin, amine (to form long chain polymers), and anhydride (to form cross-bridges between resin molecules via the hydroxyl groups) is heated together, polymerization takes place in three-dimensions, and a stable, inert substance consisting of polyesters and polyethers, very resistant to heat and solvents, forms. The length of the hydroxyl-containing part of the resin chain, the chain length of the acid anhydride, and the proportion of amine, govern the mechanical properties of the resulting polymer and affect its cutting properties. The mixed components are polymerized by heating to 60°C for 48 hours.

The cutting properties of the final polymer depend on the components of the resin monomer mixture. The mixture consists of an epoxy resin, a hardener, an accelerator (controls the rate of hardening), and a plasticizer (controls hardness of the block). No polymerization damage is seen as occurs with the methacrylates. Popular epoxy resins in use include Araldite, Epon 812 (perhaps the most widely used embedding media), DER-334 (a Dow epoxy resin), and Maraglas.

3) Embedding procedure

Tissue blocks are infiltrated with embedding medium in the same way as dehydration is performed. The final dehydrating fluid is replaced with a 50-50 mixture of dehydrating agent and resin mixture before infiltrating with 100% resin mixture. For example, with Epon 812, a stock mixture of the resin and the two hardeners is made up in the correct proportions, leaving out the catalyst. The catalyst is then added at the time the infiltration is done. The complete infiltration step usually requires several changes of embedding medium over a period of several hours.

After infiltration, the sample is usually transferred with the tip of a wooden toothpick to the bottom of a gelatin drug capsule and overlaid with the embedding mixture. The block is then hardened in an oven for 2-4 days. Special polyethylene capsules (or rubber embedding molds) with the ends already pointed into truncated pyramids help simplify the subsequent trimming procedure.

4) Block hardness

Much of the success in cutting ultrathin sections lies in matching the hardness of the embedded tissue to the hardness of the block of embedding resin. If the tissue is too hard relative to the polymerized resin, it may pull out of the soft block during sectioning. If the tissue is too soft, sectioning tends to be uneven. Epon 812 is very convenient because the proportions of the two hardeners used in the monomer mixtures control the hardness of the final block.

e. Microtomy

1) Ultramicrotomes

An ultramicrotome consists of a horizontal bar, to the front of which is attached the specimen holder (chuck) (Figs. II.14 and II.15). The bar is moved forward by means of an advance mechanism. A knife mount is positioned in front of the specimen. Sections are cut by repeatedly moving the specimen past the knife-edge with a very small advance of the specimen towards the edge made between each successive cut. Section thickness is determined by the magnitude of the forward advance of the microtome. The advance mechanism is typically thermal though early microtomes used mechanical (hand operated or by motor drive) devices.

There are many commercially available microtomes. All employ designs with a bypass mechanism to avoid specimen knife contact on the up or return stroke. Some models employ an electromagnet to pull back the knife holder assembly by about 25 μm, enabling the specimen to bypass the knife without rubbing against it on the return stroke.

To cut thin sections both reproducibly and efficiently, an ultramicrotome must meet the following requirements:

- All movements must be free of vibrations of magnitude ~10 nm or less.

- The advance mechanism must be free of static friction to permit evenness and continuity of the knife's cutting movement.
- The incremental advance of the specimen to the knife should be adjustable down to ~10 nm.
- The specimen should pass the knife-edge only once. That is, during the return phase of the sectioning cycle, the clearance between the knife and the specimen should be such as to insure the face of the block is not compressed by rubbing against the back of the knife on the return stroke.

The condition of the cut thin section depends largely on the response of the specimen block to the strains to which it is subjected while actually being cut. The ideal embedding medium is one that absorbs all extraneous strains elastically and recovers completely after the section is cut.

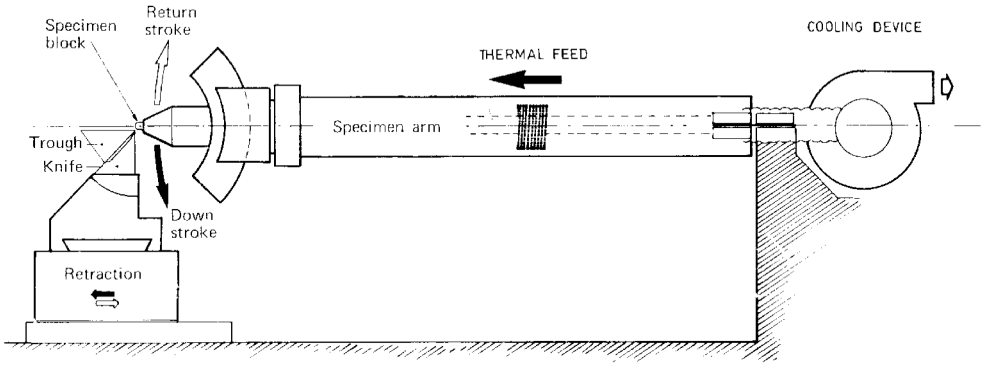


Fig. II.14. The specimen arm in the LKB Ultrotome is moved up and down along the same path, but the knife is retracted during the upward stroke until the specimen block has cleared the knife-edge (side view). (From Reid, 1975, p.227)

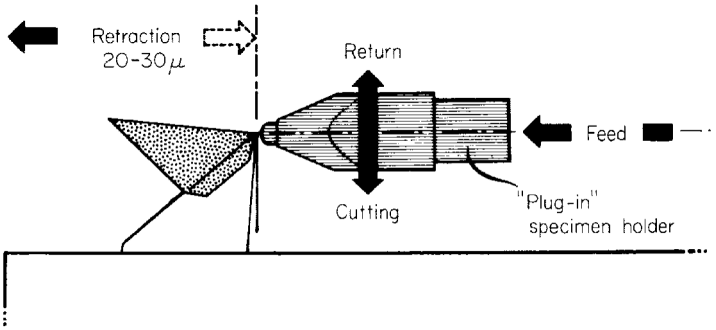


Fig. II.15. Movements of the cutting cycle of the LKB Ultrotome. The knife retracts during the upstroke and the specimen rod undergoes an up-and-down movement to cut sections while the specimen block is fed forward. All these movements are confined to one plane, that of the paper. (From Sjostrand, 1967, p.247)

2) Block trimming

The block is usually mounted in a chuck and the rounded end of the embedded block is trimmed freehand with a razor blade to a four-sided truncated pyramid of about 45° angle and about 0.5 mm square face (Figs. II.16-II.18). The smaller the area of the block face, the easier it is to cut very thin sections. Commercially available machines can be used to give precise trimming of the block. If the top and bottom edges of the block face are not exactly parallel, it may be impossible to obtain a ribbon of sections or the ribbon will curve over in one direction or another (see Fig. II.37 later) and cannot be mounted on a straight slot grid or holder.

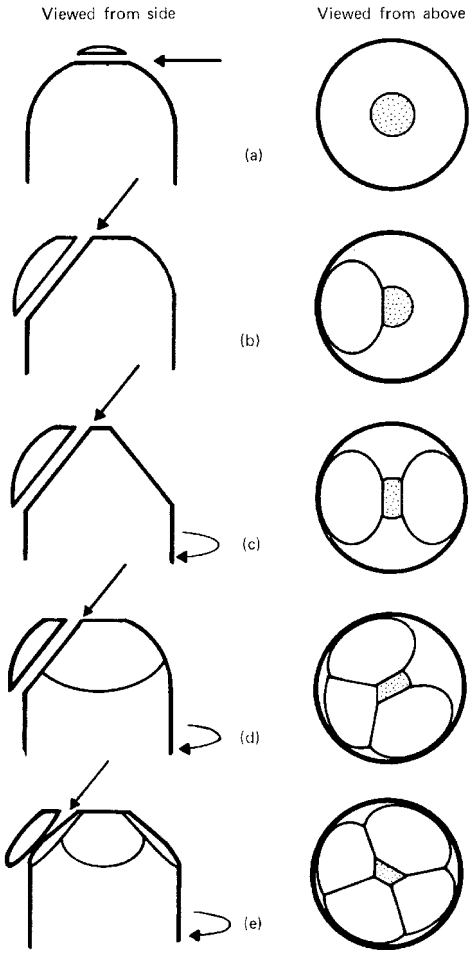


Fig. II.16. Stages of trimming of a specimen block. The straight arrows indicate the directions of cutting with the trimming blade. (From Reid, 1975, p.278)

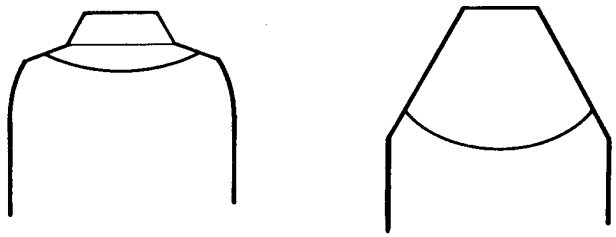


Fig. II.17. Different methods of trimming a specimen block. (a) The sides are cut in a step. (b) The cuts are continued to the sides of the block. (From Reid, 1975, p.279)

3) Knives

The characteristics of an ideal knife for ultrathin sectioning include:

- The radius of curvature of the edge is considerably smaller than the thickness of the thinnest section required.
- Resistant to chemical decomposition.
- Possess a degree of hardness and toughness that makes it impervious to cleavage or chipping on impact, even with hard blocks.
- Made of homogeneous material with the edge of same quality everywhere along its edge.
- Physically stable so as not to be subjected to net molecular migration near room temperature.

Single crystal diamond knives approach this ideal. Originally, sharpened steel razor blades were used for thin sectioning but these knives had relatively short useful life spans and their edges were subject to corrosion by the trough liquid and room atmosphere. Glass knives have proven to be the most popular of the cutting edges because they are inexpensive, relatively easy to make and are convenient to use.

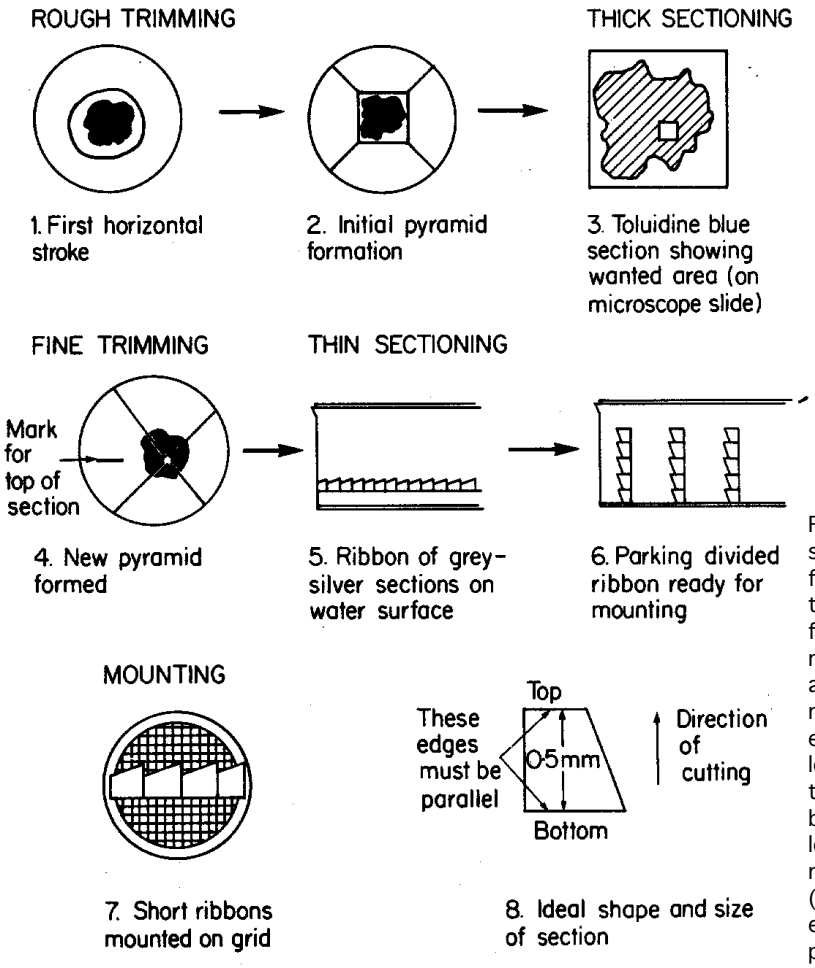


Fig. II.18 Stages in obtaining ultrathin sections. Rough block trimming (1,2) is followed by the localization of the required tissue area in a 0.5 mm thick section stained for the light microscope (3). The block is retrimmed to the size and shape shown in (8), and a ribbon of grey sections is cut (5). The ribbon is divided up, using the finely pointed ends of mounted eyelashes, into short lengths, which are 'parked' against the side of the trough (6). A TEM grid is then introduced beneath the water surface, and each short length of sections is drawn on to a grid by means of an eyelash. The mounted sections (7) are then stained before examination in the electron microscope. (From Meek, 1970, p.453)

A glass knife is generally triangular in shape, and is obtained by breaking a square piece of glass along a diagonal line scored into its surface. The cutting edge should be straight and even and the front surface (facing the specimen) very flat and smooth. The polished, fractured edge of a diamond knife is harder and more wear resistant than glass, but it is not a better cutting edge. Also, the diamond knife must be cleaned before each use (Fig. II.19).

Making good glass knives is the single most important step in obtaining good sections. The knives can be made by hand (only with much experience) or using a special machine that helps give reproducible results (*i.e.* length and position of score, depth of score, position of the bending force, amount of the bending force, and the rate at which it is applied) (Fig. II.20).

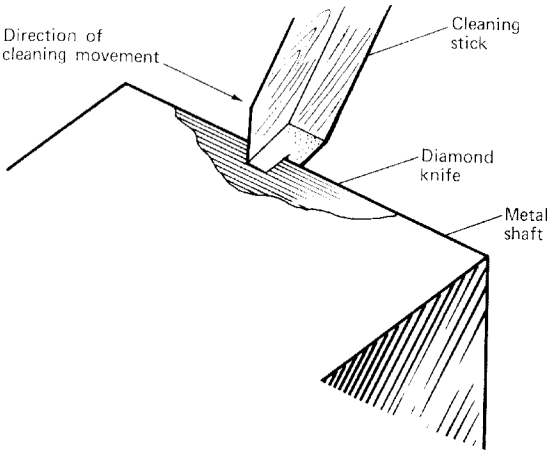


Fig. II.19 Cleaning a diamond knife with a soft wood stick. All movements must be made parallel to the knife-edge. (From Reid, 1975, p.266)

Fig. II.20. The LKB knife maker. (From Sjostrand, 1967, p.270)

Knife position

The position of the knife, relative to the tissue block, is critical if uniformly thin sections are to be cut. The positional relationship of the knife-edge to the specimen involves several angles defined as follows (Fig. II.21):

Rake Angle - angle between the line perpendicular to the front of the face of the block and the upper facet of the knife-edge.

Knife Angle - angle subtended by the rear and upper facets of the knife-edge. Also known as the **bevel angle.**

Clearance Angle - angle between the rear facet of the knife-edge and the vertical plane of cutting.

The usual knife angle is about 45° and the clearance angle is commonly adjusted to 2-5°.

The Knife Trough

Thin sections have little physical strength. If they are cut on a dry knife, the static friction between the facet and the cut surface almost always causes the section to wrinkle as it is cut. Even so, the chances of picking up the section onto a metal grid undamaged and undistorted are very slim. The use of a trough or "boat" of liquid at the cutting edge provides a low friction surface for support of the freshly cut section. It also simplifies picking up the section on specimen support grids.

The simplest way to prepare a trough is with the use of some waterproof tape (*e.g.* silver tape) positioned so it doesn't overlap the cutting edge or the front face of the knife. Melted dental wax is used to seal the trough against leaks. Manufactured metal troughs are also available. For diamond knives, the trough is an integrally cast part of the holder (Figs. II.22-II.24).

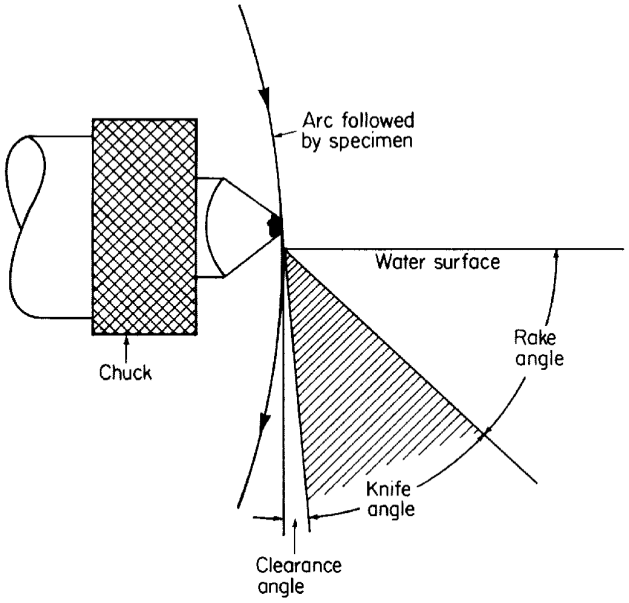


Fig. II.21 Knife and cutting angles. The clearance angle is tangential to the arc followed by the specimen. The knife angle does not necessarily bear any relationship to the actual cutting angle at the specimen. (From Meek, 1970, p.448)

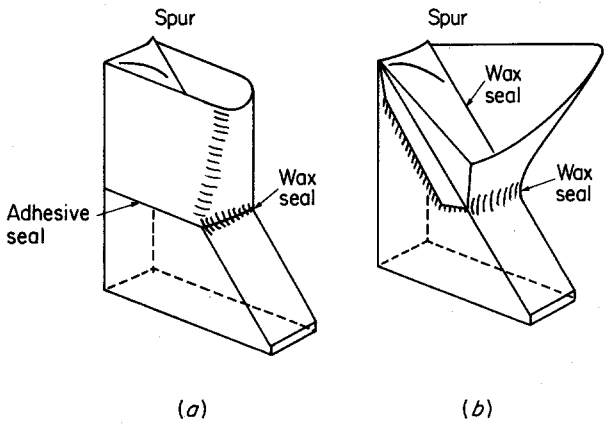


Fig. II.22 (a) An adhesive tape trough waxed to the knife. (b) A metal trough waxed to the knife. The metal trough has a greater water surface area for manipulating ribbons of serial sections. (From Meek, 1970, p.445)

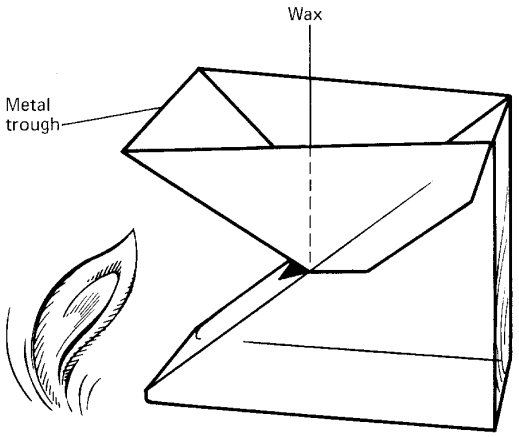


Fig. II.23 A glass knife fitted with a metal trough. The back of the trough is gently warmed to melt the wax inside and form a watertight seal. (From Reid, 1975, p.263)

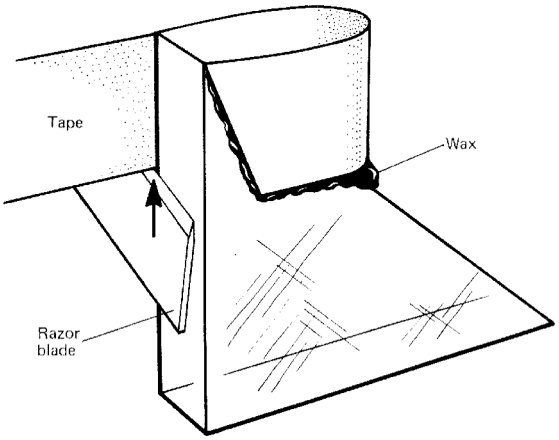


Fig. II.24 A tape trough being applied to a glass knife. The razor blade is only used to cut the tape on the side furthest from the usable region of the knife-edge. (From Reid, 1975, p.264)

4) Cutting thin sections

The trimmed block is mounted in the microtome chuck. If the chuck is tightened too much the plastic will be squeezed out and flow towards the knife causing ultrathick sectioning. If the block is held too loosely it may vibrate and cause chatter marks on the sections. The block should also be set as far into the chuck as possible with little overhang to reduce chatter. The top and bottom of the block face should be set parallel to the knife-edge (Figs. II.25 - II.27). In setting up the knife, the most important angle is the clearance angle, which should be small, but large enough so the block face does not contact the front face of the knife after the section is cut. The normal angle is between 1 and 5°. The softer the block, the greater the clearance angle must be.

Once the block face is parallel to the knife-edge, the trough is filled with water exactly to the level of the knife-edge, leaving no meniscus. The knife is carefully advanced to within a few microns of the specimen block face. This maneuver takes experience to master without ending up too far away or so close that the knife hits the specimen. The automatic advance of the ultramicrotome is

then activated to initiate the thin sectioning. Alternatively, on manual microtomes, the operator, who determines the thickness of each section by setting the advance mechanism to the desired position, initiates each cycle of sectioning. If everything has been set up correctly, a ribbon of thin sections should float from the knife-edge into the trough (Figs. II.28-II.29).

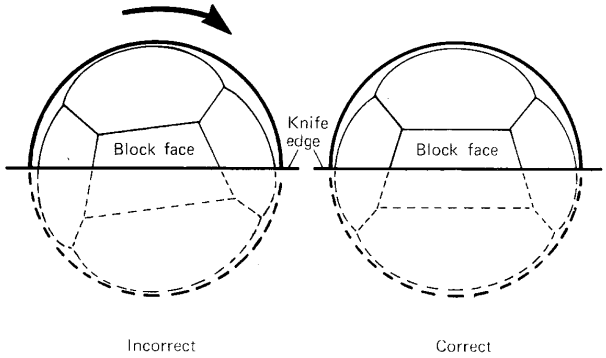


Fig. II.25 The specimen block is oriented so that the upper and lower edges of the block face are parallel to the knife-edge. The broken lines indicate the part of the face that is behind the knife when viewed from the front. (From Reid, 1975, p.290)

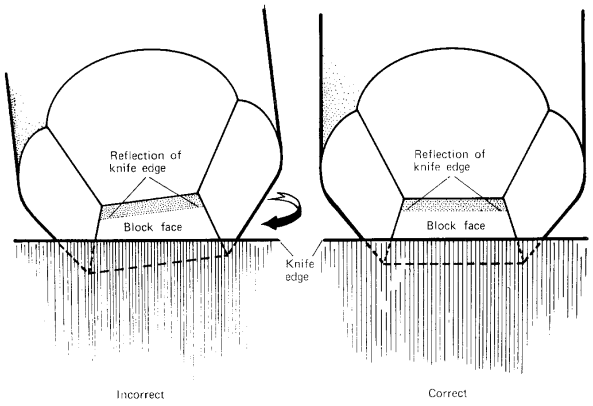


Fig. II.26 To ensure that the whole of the block face is cut, the specimen holder is adjusted until the knife-edge is parallel to its reflection seen in the mirror-like block face. (Viewed at a small angle from the vertical.) (From Reid, 1975, p.291)

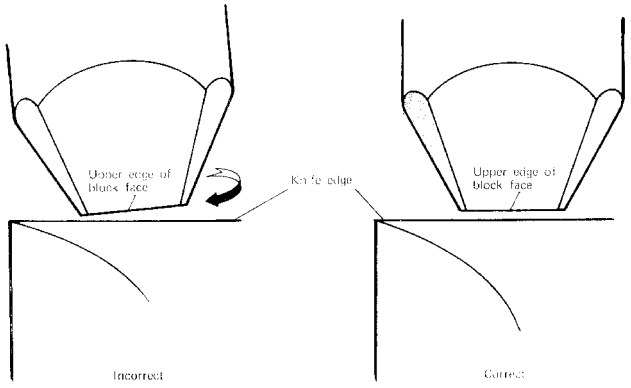


Fig. II.27 When viewed from above, the upper edge of the block face should be parallel to the knife-edge. (From Reid, 1975, p.293)

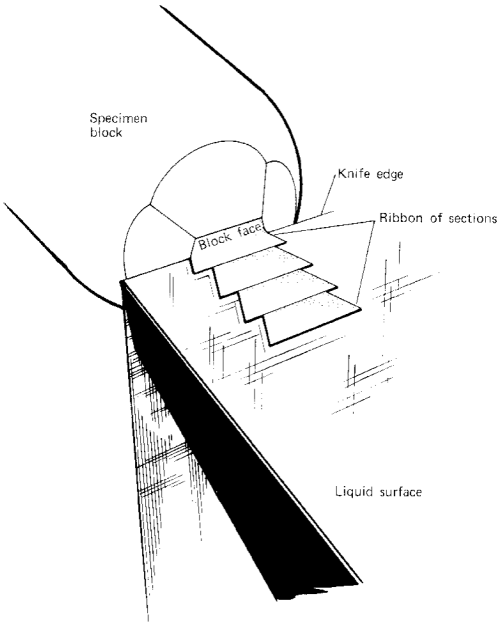


Fig. II.28 Sections adhering together in ribbon formation while floating on a liquid surface. (From Reid, 1975, p.222)

5) Flattening compressed sections

The stress in compressed sections (Fig. II.30) is often relieved by exposing the sections to the vapor of a solvent such as xylene or ethylene dichloride, applied by holding a small brush dipped in the solvent close to the surface of the section as it floats on the water surface. Sometimes a little ethanol or acetone is added to the water in the trough to help release compression strains or distortions introduced during sectioning. Surface tension acting on the cut section is able to stretch out the section to the original dimension of the block face.

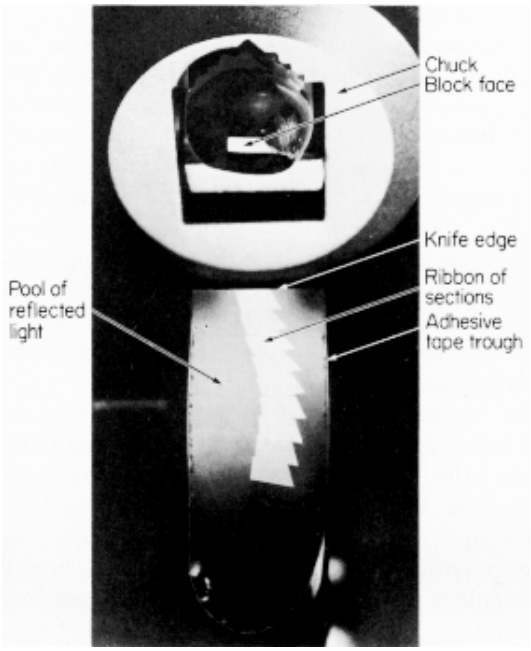


Fig. II.29 Appearance of sections floating on the water surface in the trough when illuminating conditions are correct. (From Meek, 1970, p.451)

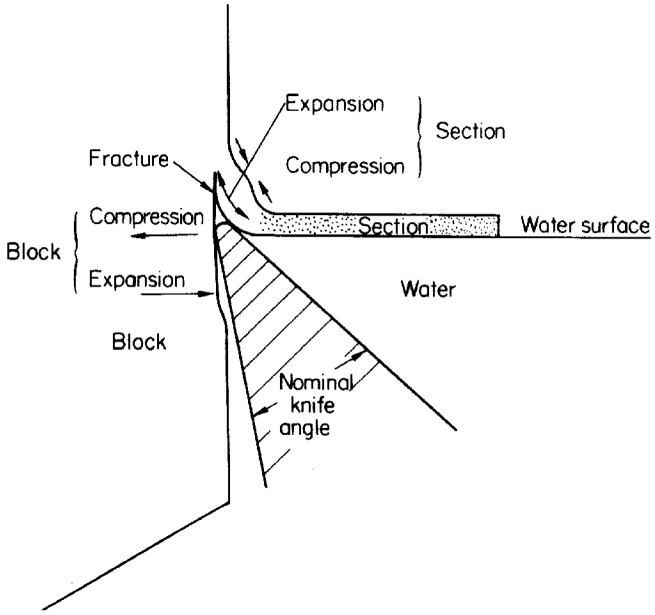


Fig. II.30 Stresses suffered by the section and block during cutting. The actual cutting edge is curved, and fractures the block before paring the section off. The section may be considerably compressed, especially if the knife-edge is blunt. (From Meek, 1970, p.449)

6) Mounting sections on grids

The most common procedure for transferring thin sections to the EM grid is to insert the grid, held with a pair of forceps, under the water surface and pick up the floating sections from below (Figs. II.31 and II.32). The fragile sections will generally wrinkle if they are picked up from above.

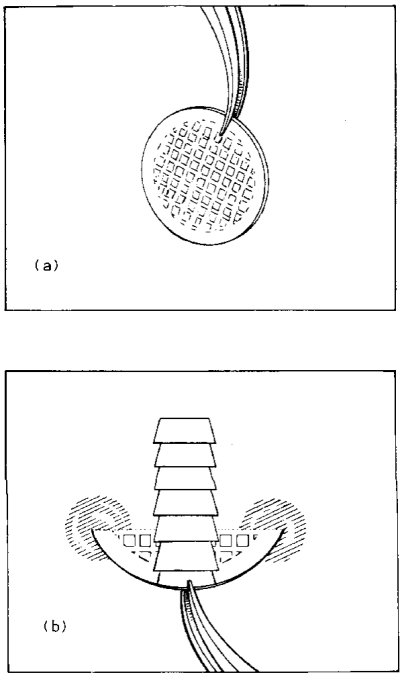


Fig. II.31. (a) A specimen support grid held in a pair of TEM forceps. (b) The grid partly removed from the water with one end of the ribbon of sections adhering to it. (From Reid, 1975, p.315)

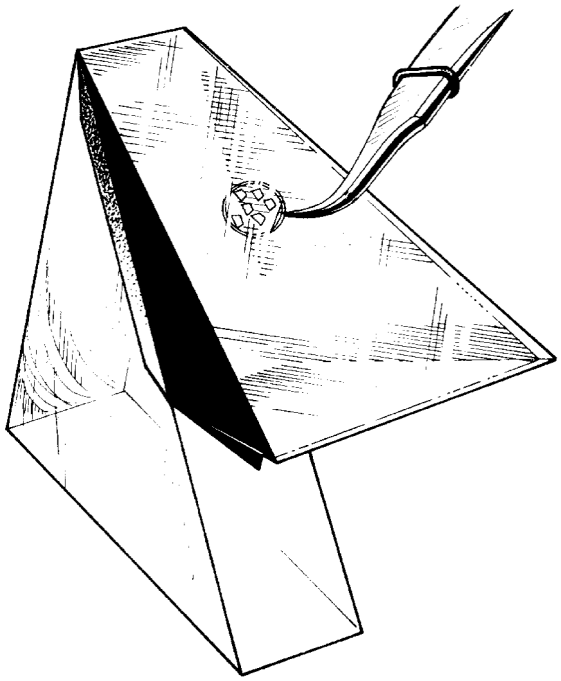


Fig. II.32. A cluster of single sections being collected by raising the grid underneath them. (From Reid, 1975, p.316)

7) Section thickness

All modern ultramicrotomes have automatic advance mechanisms. It is generally difficult if not impossible to cut sections of uniform thickness much thinner than 30-40 nm. The thickness of sections can be estimated from their color in reflected light. The difference in path length between the light reflected from the surface of the water beneath the section and the light reflected from the upper surface of the section gives rise to interference between the two light rays, which, if white light is used, causes reinforcement at certain visible wavelengths and hence makes the sections appear to be colored. The thinnest sections (<60 nm) appear dark grey to grey in color. Thicker sections appear silver (60-90 nm), gold (90-150 nm), or purple (150-190 nm).

The quality of the section cut is primarily dependent on the quality of the knife-edge and the dimensions and shape of the specimen block. A dull or chipped knife, inadequate clearance angle, and too large a block face may result in faulty sectioning. With a good knife edge that is properly positioned and with a well trimmed block, the quality of the section is dependent on the physical properties of the embedded block that affect its behavior when it is under strain during the actual cutting process.

f. Section staining

Thin sections are usually stained with solutions of heavy metal salts to enhance the scattering contrast of specimens by increasing the mass density differences of various components of tissues and cells, thus increasing the scattering of electrons outside the objective aperture. The metal ions of the staining solutions form complexes with certain components of cells, thus increasing their density. Often, such staining has little chemical specificity, but the contrast of components such as ribosomes and membranes is increased relative to their surroundings.

The minimum thickness for visibility of a specimen is a function of the thickness of the object (in nm) times its density (g/cm^3) and must be about 10 or greater. An unstained biological specimen has a density of about 1g/cm^3, thus it must be at least 10 nm thick to be barely visible relative to its surroundings.

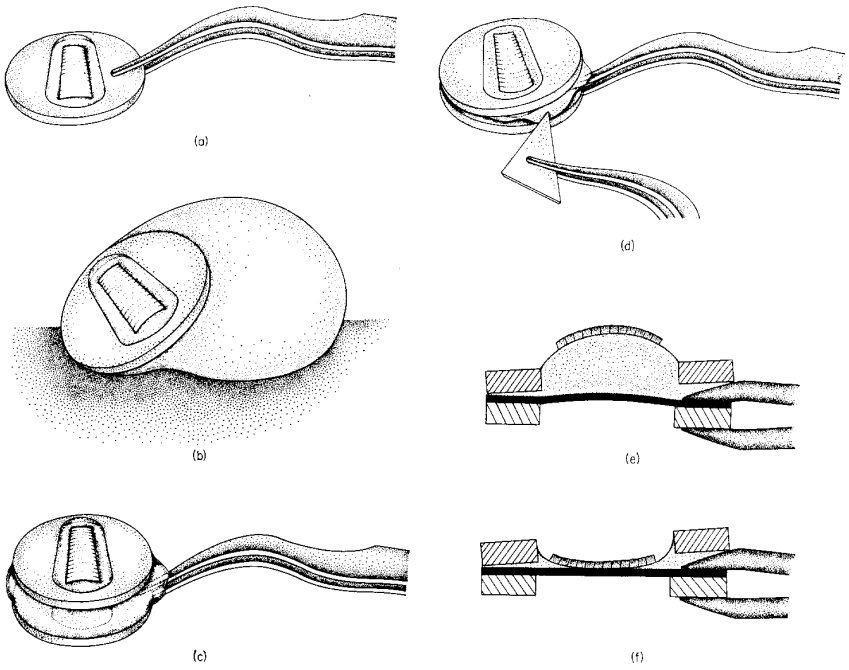


Fig. II.33. Transferring ribbons of sections to staining solution and to film-covered one-hole ('slotted') microscope grid by means of a similar slotted grid. A ribbon slightly shorter than the hole of the grid is broken off from the knife-edge and moved to the center of the trough. (a) The ribbon is picked up by means of a one-hole grid with no supporting film, placed on the surface of the trough with the ribbon in the center of the hole. The grid is picked up from the water surface by means of a pair of forceps. The ribbon stays in the center of the hole on the drop of water adhering to the grid. (b) The pick-up grid is placed on a drop of a staining solution in a Petri dish with wax-covered bottom. (c) Pick-up grid is then transferred to a similar grid with supporting film after proper washing by transferring the pick-up grid to a surface of distilled water. A water drop separates the two grids. (d) Removal of the water by means of a small piece of filter paper. (e) and (f) When the water is removed, the ribbon sinks down through the hole of the pick-up grid and eventually is collected on the supporting film of the second grid. It is important that the two grids are prevented from making contact over any large surface area by means of the pair of forceps holding the second grid. (From Sjostrand, 1967, p.292)

A variety of stains are available, with the most useful employing lead or uranium ions. The most commonly used heavy metal for section staining is lead (as lead citrate). 1% uranyl acetate in

water or alcohol is the other most commonly used stain (alcohol appears to promote more rapid staining). Because of the different staining properties of uranium and lead, it is common practice to double-stain sections, first using warm uranyl acetate (30 min) followed by lead citrate (1-2 min). Other common stains include phosphotungstic acid and potassium permanganate and other lead (*e.g.* tartrate, cacodylate, citrate and ammonium acetate) and uranyl salts.

The section is reacted with a solution of a heavy metal salt such as lead citrate by floating the sections mounted on the grid on the surface of a 50-100 μl droplet of the staining solution, usually for 10-60 minutes (Fig. II.32). The single droplet of staining solution is placed on the surface of a piece of Parafilm or dental wax and the grid is placed with the sections facing the surface of the drop. The grid is then removed and the sections washed with distilled water to prevent stain crystals from contaminating the surface of the section when it dries.

g. Sectioning artifacts

Unsatisfactory sections arise either from faulty embedding procedures or from faulty cutting. Section faults must be clearly distinguished from artifacts of faulty tissue preservation. Faulty sections are almost invariably due to use of bad knives, improper knife angle, poor infiltration or polymerization, incorrect block hardness or lack of skill on the part of the operator (Figs. II.34-II.36).

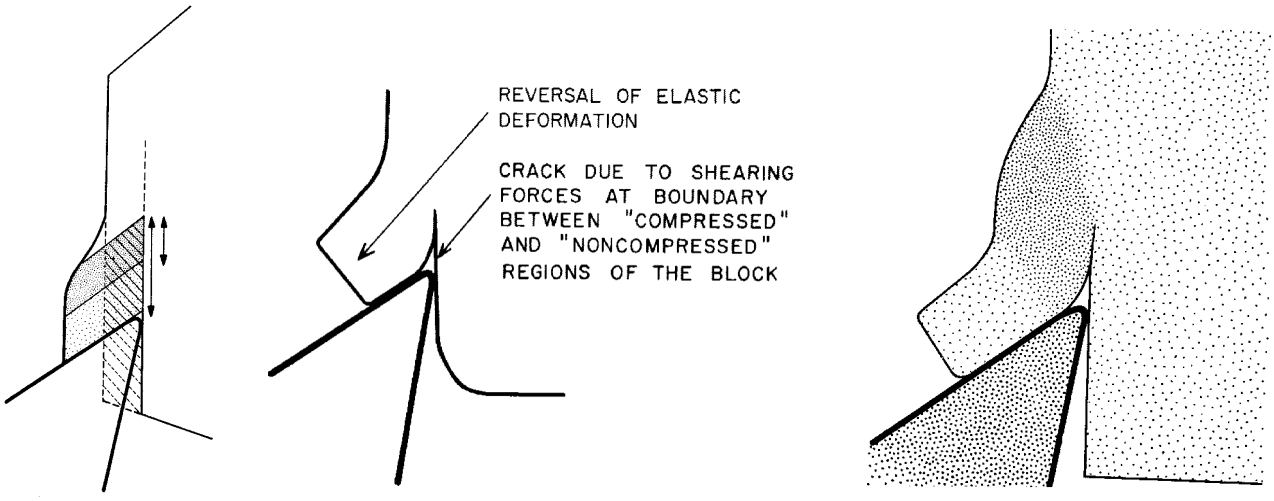


Fig. II.34 (Left). Displacement of the embedding medium due to plastic and elastic flow as a basis for splitting off of a thin surface layer of the specimen block. Arrows indicate the reduction of the dimension of this surface layer in the direction of the cutting. (Middle). Theory of ultrathin sectioning. The displacement of the surface layer of the block introduced by the knife is partially reversible (elastic deformation). (Right). Theory of ultrathin sectioning. A zone of maximum deformation is indicated by the darkest stippling. (From Sjostrand, 1967, p.233)

If sections cling to the upper edge of the block when cut and disappear down with it, the cause is probably due to the block pyramid being trimmed with a blunt razor blade. The pyramid sides must be mirror smooth or the ragged surface will cause tiny threads to remain between the section and upper edge of the block face. The same problem occurs if the block face has become wet.

If the sections alternate between thin and thick, the cause may be a blunt knife, a bad match between block and tissue hardness, poor infiltration, or irregular polymerization due to inadequate mixing of the resin components. Skipping, in which a section is cut, then no section, etc., means that cut sections are twice the thickness desired. This may result from the advance being set too small.

Chatter, or alternating lines of thick and thin areas of the section parallel to the cutting edge of the knife are caused by vibration of the knife or the block or both during sectioning (Fig. II.37). The block may be protruding too far out of the chuck or it may not be firmly gripped. The knife may also not be firmly gripped or the clearance angle may not be set correctly or the cutting speed may be set too high.

Scratches running in the cutting direction arise from imperfections or chips in the cutting edge of the knife. Either a new portion of the knife-edge must be used or the knife should be replaced.

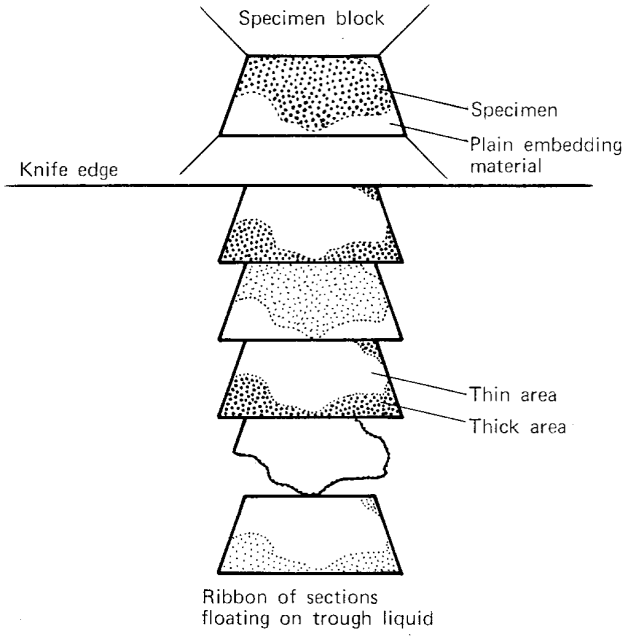


Fig. II.35 Variations in thickness seen as straight bands parallel to the knife-edge are caused by vibrations. (From Reid, 1975, p.309)

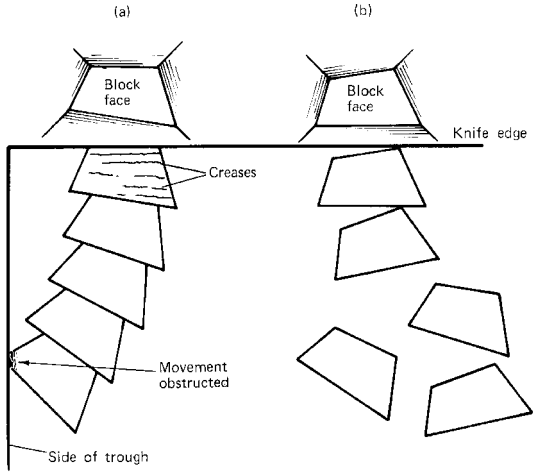


Fig. II.36 The upper and lower edges of the block face are not parallel to one another. (a) The ribbon of sections has curved towards the side of the trough and become obstructed. (Right) When the top edge of the block is not parallel to the knife, the sections are only in contact with each other at one point and quickly float apart. (From Reid, 1975, p.301)

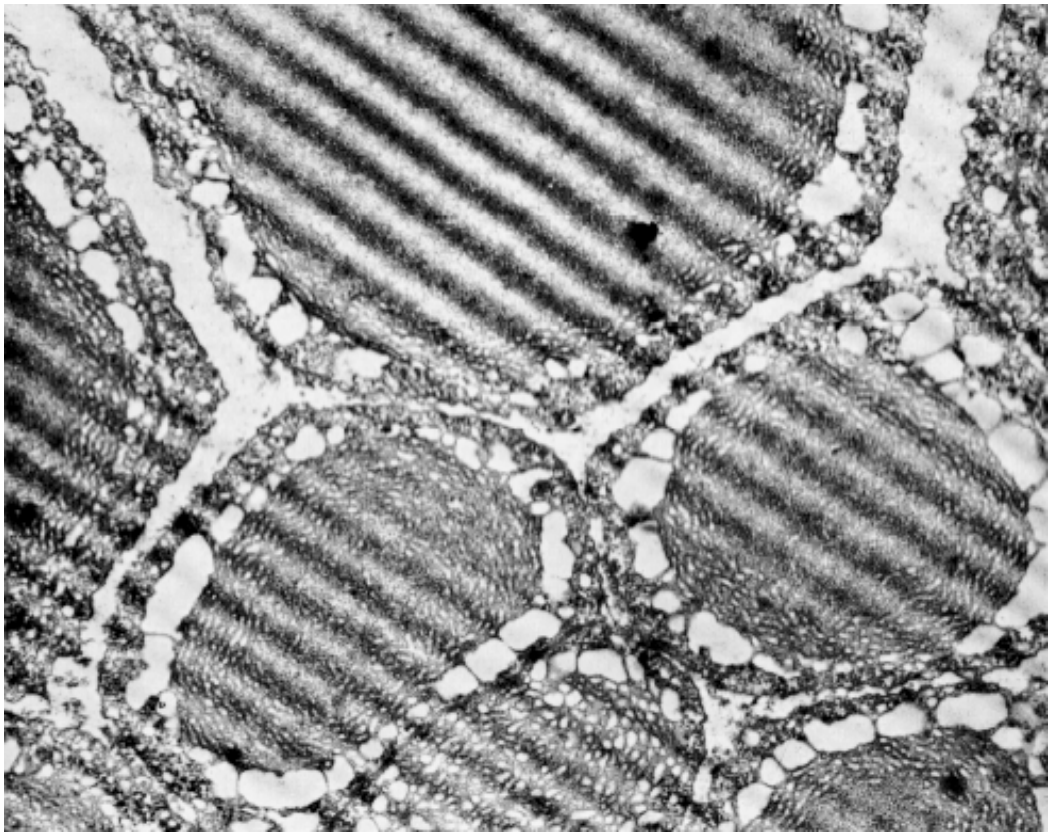


Fig. II.37. Thin section "chatter". Periodic variation of section thickness is caused when the block tip vibrates after impacting the knife. (From Sjostrand, 1967, p.235)

II.A.3. Negative-Staining

a. Introduction

For structural detail to be recorded in a micrograph, it must be faithfully preserved as the specimen is prepared and subsequently exposed to the vacuum and electron beam. The main purpose of negative staining is to surround or embed the biological object in a suitable electron dense material that provides high contrast and good preservation (Fig. II.38). This method is capable of providing information about structural details often finer than those visible in thin sections, replicas, or shadowed specimens. In addition to the possibility of obtaining a significant enhancement of contrast, negative staining has the advantage of speed and simplicity.

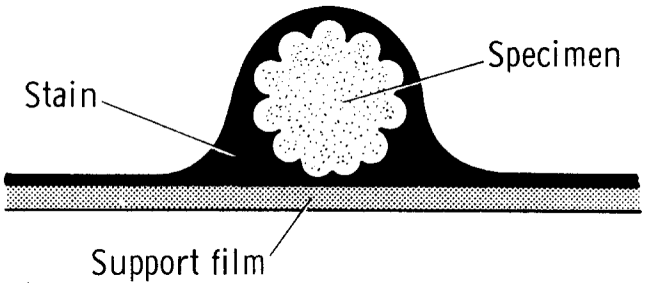


Fig. II.38. Schematic representation of a specimen particle completely embedded in a negative stain. (From Hayat and Miller, 1990, p.2)

The potential value of negative stain microscopy derives from the relative ease with which specimens can be imaged at the molecular level of resolution. The technique has mainly been used to examine particulate (purified) specimens - *e.g.* ribosomes, enzyme molecules, viruses, bacteriophages, microtubules, actin filaments, etc. at resolutions of 1.5-2.5 nm. This technique generally allows the shape, size, and the surface structure of the object to be studied as well as provide information about subunit stoichiometries and symmetry in oligomeric complexes. Any surface of the specimen accessible to water can potentially be stained, and thus, that part of the specimen will be imaged at high contrast.

b. Historical notes

Hall (1955) was the first to accidentally demonstrate the negative staining effect in a study in which particles were being positively stained with phosphotungstic acid. Imperfectly washed particles were surrounded and embedded in the dried reagent and, instead of appearing dark on a light background, they were seen light on a dark background. Huxley (1957) independently noticed the same effect in a study with tobacco mosaic virus. Brenner *et al.* (1959) also observed the same phenomenon with T2 bacteriophage and were the first to call the process **"negative staining".**

c. Enhancing contrast of biological specimens

An object, whatever its chemical composition, under normal conditions of TEM will barely be discerned (*i.e.* just noticeable contrast) in the electron image if the product of its thickness (in nm) and its density (in g/cm^3) is more than 10. Biological specimens have an average density of just slightly above $1 g/cm^3$, thus it is difficult in a micrograph to discern specimen features any smaller than 10 nm without some treatment to enhance contrast. Positive staining involves treatment of the specimen with a chemical that increases specimen density. However, even intense staining that doubles the density would still only allow objects larger than 5 nm to be seen. When positively stained, the object of interest appears in the image as an electron-opaque area against a relatively light background. Alternately, the electron-transparent object is “negatively stained” if it is more or less completely embedded in a matrix of electron dense-material, and appears in reversed contrast as an electron-transparent area against a dark background. Contrast enhancement by positive staining involves a direct interaction of a stain material with the protein. Negative staining does not require a stain-protein interaction, and denaturation of the molecule by staining is minimized, yet visualization to a limit of resolution of about 2-2.5 nm is easily realized.

d. Properties of an ideal negative stain

Each of the commonly used stains fails in some respect to meet the "ideal" specifications outlined below. An ideal stain should have:

- High density

- High solubility
- High melting and boiling points
- Amorphous structure (*i.e.* structureless) when dry
- Ability to protect specimen against dehydration effects
- Non-chemically reactive with the specimen
- Uniform spreading on the support film

High weight density to give high contrast

The density of most biological specimens varies between 1.0 (lipids) and 1.7 g/cm^3 (nucleic acids). To increase specimen contrast, mass thickness must be increased. The addition of non-specific stain to the specimen increases the specimen mass thickness, the effect being greater, the greater the mass density of the stain. Ideally the stain should be of uniform thickness and just sufficiently deep to completely engulf the specimen molecules. The "whole specimen" should be of limited thickness (10-50 nm). The resulting contrast of the specimen is much lower than that of the embedding amorphous stain film. In the photographic **negative**, the specimen appears dark against a light background**.** Hence the term "negative staining", which is not staining at all, but is embedding and is "negative" only in the sense that the biological material has less contrast than the surrounding stain.

The density of most dried stains ranges between 3.8 and 5.7 g/cm^3. The actual chemical composition of the stain has little effect on contrast. Note that the degree of contrast in the immediate vicinity of the object is determined by the density of the contrasting ion, not by the density of the contrasting substance as a whole. Heavy metal stains mostly cause elastic scattering of electrons, thus the main process of contrast formation is that of scattering contrast**.**

High solubility

The stain must be sufficiently soluble to allow enough to dissolve and come out of solution only in the final stages of drying. A relatively insoluble salt comes out of solution well before the drop finally dries (§ II.A.3.g) and deposits as crystals without outlining or penetrating the specimen.

High melting and boiling points

The stain must be reasonably stable under the conditions to which the specimen is exposed in the microscope so it does not run or volatilize when heated by the electron beam. Energy transferred by inelastic scattering events produces radiation damage in the specimen. The rupture of chemical bonds with the formation of new structures and the ejection of materials from the irradiated area will eventually degrade the specimen. Transfer of energy to the specimen may also result in thermal degradation of the specimen and produce image drift due to thermal stresses in the specimen. Useful stains are highly resistant to such attack. Since the ultrastructural detail observed is a consequence of the distribution of stain, any degradation of the stain deposit during exposure to the beam will result in the loss of detail in the image.

Amorphous (non-crystalline) when dry

The drying of the stain and specimen, together with any buffer, may produce contrast unrelated to the structure under study. Such "noise" degrades the quality of the image and may be of sufficient magnitude to obscure structural detail. To be most effective, the stain must deposit in an amorphous bed of uniform thickness and density to produce an artifact-free background against which the images of the specimen are viewed. A tendency to form microcrystals or other anomalies of structure or density in an otherwise homogeneous deposit will result in excessive "grain" effects in the slightly underfocused micrograph. Contrast from these effects may obscure the fine morphological details in the specimen.

If the molecular dimensions of the hydrated stain are the same or larger than the morphological features in the specimen, the visualization of those features may be prohibited. Thus, some advantages may accrue from the use of stains of low molecular weight. Stains such as phospho-tungstate and silicotungstate have dimensions of the order 0.8-0.9 nm whereas uranyl stains are considerably smaller (0.4-0.5 nm). This limits the possible resolution of specimen detail to values that are seldom if ever below 0.7 nm. Under normal conditions, resolution is limited to about 2 nm

by inherent noise in the electron image. The highest resolution possible is obtained with negative stains such as uranyl formate, which are characterized by smaller size and hence increased penetrability. Less penetrating stains such as phosphotungstate are often used advantageously in systems where extensive penetration might obscure certain surface detail.

Non-reactive with the specimen

A desirable and sometimes essential property of the stain is that it be chemically inert**.** An ideal stain is inert to the specimen as well as to the buffers, salts, metal ions, cofactors, or other reagents commonly used to maintain the integrity of the specimen structure. It is soluble and stable over a wide range of pH values. Any one stain may prove unsuitable for use with a particular specimen preparation by virtue of its reactivity with the specimen or the buffer solution. Uranyl salts are unstable at pH values >6 and are generally used below that pH. Uranyl ions form an insoluble compound with phosphate ions. Hence, use of phosphate buffers in samples may lead to reaction between buffer and negative stain.

Partial disruption or changes in the tertiary structure of specimen molecules may occur with negative staining. This may prove advantageous or it may be necessary to chemically fix the specimen before staining to prevent disruption. Low concentrations (0.5-2%) of glutaraldehyde are sometimes used to fix the specimen before staining. To prevent inter-particle cross-links, the fixation is carried out for short periods (generally < 30 min) with extremely dilute samples. Another useful cross-linking agent is the bifunctional reagent dimethylsuberimidate, which preserves positive charges at lysine residues in proteins, thus reducing conformational changes.

Protect against dehydration effects

In the final stages of drying, the dense stain ideally forms a uniformly thin, amorphous film in which the specimen is supported and preserved (Fig. II.39). In theory, stain replaces water in the interstices of the object and, in the dried specimen, all previously hydrated volumes are filled with it. The enveloping stain then supports the specimen structure, if the deposit of stain is thicker than the embedded specimen. The specimen thus escapes exposure to surface tension forces and hopefully, retains its native morphology. A material of limited solubility may begin to precipitate before the last stages of drying and fail to engulf and protect the specimen. In general, those materials that make the best stains are very soluble.

Considerable changes in salt concentration and pH accompany drying of the specimens, but the stain helps protect the specimen from serious dehydration damage. When the specimen molecules are dried within a matrix of negative stain, surface tension forces are dissipated against the stain bed surface, thereby minimizing distortion of the specimen ultrastructure.

Uniform spreading on the support film

Excellent and uniform wetting of the support film surface is necessary to consistently achieve micrographs of good quality. A hydrophilic film surface enhances the tendency of the stain solution to spread and deposit the stain in an acceptably thin bed, free of artifacts (Fig. II.39). The support film may be made sufficiently hydrophilic either by glow discharging the grids or by treating the surface of the film first with a dilute (0.1 mg/ml) solution of cytochrome c or serum albumin before applying the specimen sample.

e. Additional notes about negative staining

1) Specimen purity

Negative stain microscopy is an excellent technique for assessing the purity of preparations and to appraise the efficiency of preparative procedures. Negative staining is of value in purification procedures, both for establishing a criterion of purity and for locating small amounts of a particular specimen particle in a fractionation scheme.

2) One- verses two-sided staining

With negative staining, sometimes only the top or bottom of a particle is contrasted with the stain. This usually occurs if the stain layer is very thin. In thicker layers of stain, particles will generally stain on both sides. There seems to be no way to obtain reproducibly one- or two-sided images for most specimens. Also, it is often difficult to ascertain the degree to which stain embeds both sides of a particle.

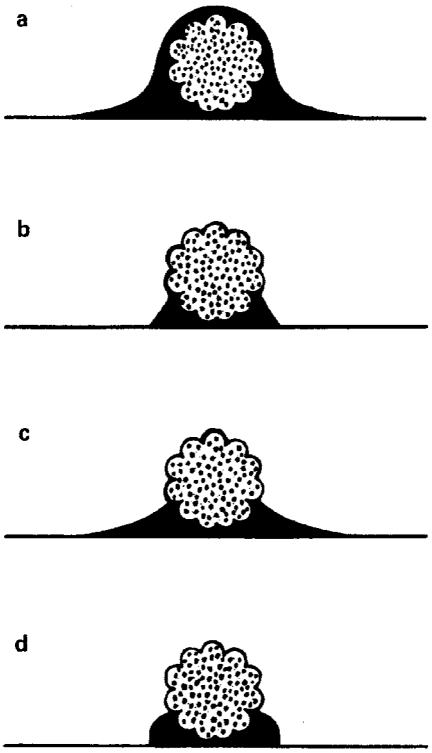


Fig. II.39. Schematic representation of the effect of the support film and the specimen surface on the meniscus of the stain before drying. (a) Hydrophilic specimen on hydrophilic support film. The adhesiveness of the stain to both the specimen surface and the support film is almost equal; it is greater than the cohesiveness of the stain. (b) Hydrophilic specimen on hydrophobic film. The adhesiveness of the stain to the specimen surface is stronger than the adhesiveness to the film, but the adhesiveness of the stain to the film is less than the cohesiveness of the stain. (c) Hydrophobic specimen on hydrophilic film. The adhesiveness of the stain to the film is stronger than the adhesiveness to the specimen surface. The adhesiveness of the stain to the specimen surface is less than the cohesiveness of the stain. (d) Hydrophobic specimen on hydrophobic film. The adhesiveness of the stain to the specimen surface is greater than stain cohesiveness, but the stain cohesiveness is less than the adhesiveness of the stain to the film. Alternatively, the adhesiveness of the stain to the specimen surface is equal to the adhesiveness to the film, but the adhesiveness to the film is stronger than the cohesiveness of the stain. (From Hayat and Miller, 1990, p.23)

3) Positive vs. negative staining

Since most biological specimens have charged groups exposed to the surrounding aqueous solvent, and the stain molecules are ions, there will almost always be some degree of positive staining (Fig. II.40). For example, phosphotungstate ions are negatively charged and they can be used most effectively as a negative stain only for specimens raised above their isoelectric point so they do not attract the contrasting ions. Uranyl acetate at pH 4.5 is used as a negative stain with specimens that are positively charged below pH 5. With the anionic (*i.e.* attracted to a positive charged material) stains such as the phosphotungstates, positive staining is expected in the region of low (acidic) pH values because of the powerful interaction between positive charges in the specimen and the negatively charged tungstate ions.

4) Maintenance of biological structure/function

It has been found that some viruses are still infectious after mixing and spraying with phosphotungstate and certain enzymes remain active even after they have dried in droplets of stain and are subsequently rehydrated.

5) Reliability of images

It is always important, especially with unfamiliar specimens, to try a variety of conditions (stain, pH, temperature, concentration of specimen, stain and buffer, etc.) when preparing specimens for microscopy using negative staining techniques. Quite often, under varied conditions, different features of a specimen will be enhanced, and either complementary or perhaps even contradictory information may be obtained. If, for example, a variety of stains are used and similar staining patterns are obtained, then it is likely that the features revealed are consistent with genuine specimen morphology.

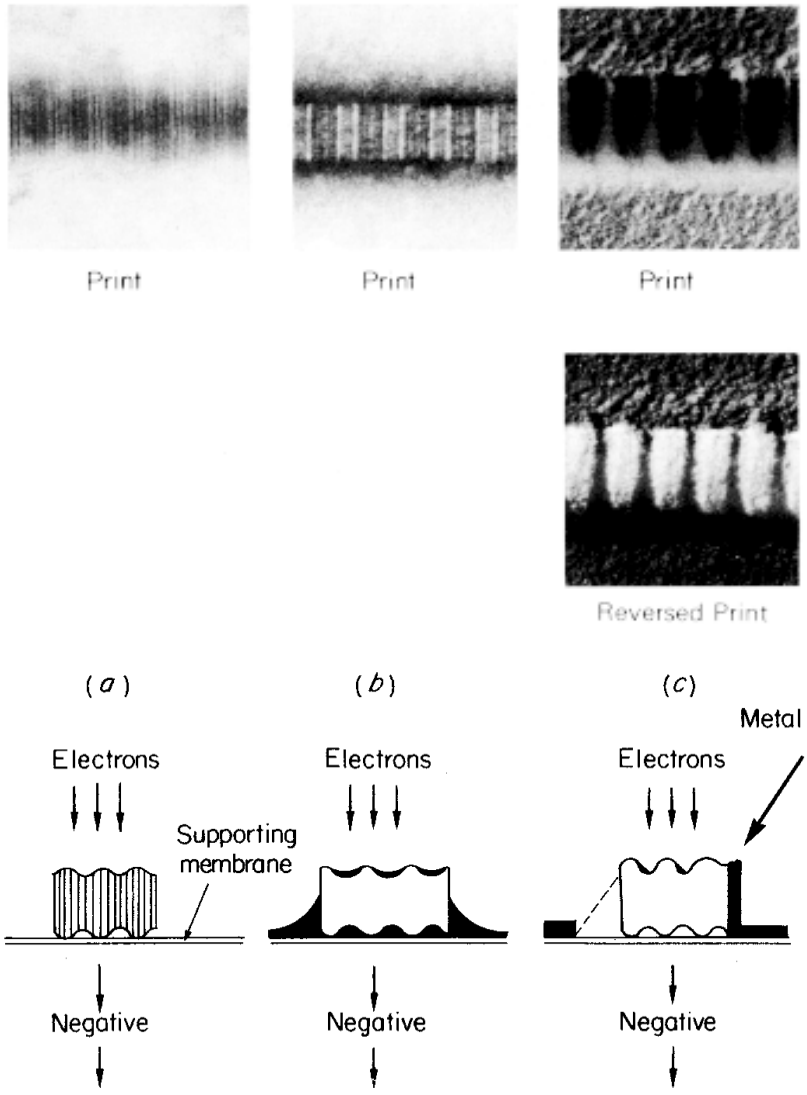


Fig. II.40. The effects produced by (a) positive staining, (b) negative staining, and (c) metal shadowing on a fragment of collagen lying on a supporting membrane. The overall effect of banding is clearly shown by all three methods, but each supplies certain information, which is lacking in the others. Shadowing shows clearly the granular nature of the carbon support film. Metal shadowing is covered in §II.A.4. (From Meek, 1970, p.466)

f. Common negative stains

The following table lists some chemicals used as negative stains along with some properties:

STAIN	FORMULA	Solubility (g/100ml H_2O)	Anhydrous Density (g/cc)
ANIONIC STAINS			
Ammonium molybdate	$NH_4Mo_7O_{24}{\cdot}4H_2O$	44	2.5
Sodium phosphotungstate	$Na_3PO_4{\cdot}12WO_3$	?	3.8
Sodium tungstate	Na_2WO_4	90	4.2
CATIONIC STAINS			
Silver nitrate	$AgNO_3$	220	4.4
Cadmium iodide	CdI_2	85	5.7
Uranium nitrate	$UO_2(NO_3)_2{\cdot}H_2O$	150	3.7
Uranyl acetate	$UO_2(C_2H_3O_2)_2{\cdot}2H_2O$	8	2.9
Uranyl formate	$UO_2(CHO_2)_2{\cdot}H_2O$	7	3.7

Oliver (1973) reports that methyl phosphotungstates and methylamine tungstate may be used as negative stains. They have the primary advantage of a greater tendency to wet the support film surface, spreading over sufficiently large areas giving a relatively thin stain bed of good uniformity. They appear to be stable over a wide range of pH values (from pH 4 to 9.5).

g. Negative Staining procedures

Techniques for the preparation of negative stain specimens are simple and direct. The essential aim of the procedure is to embed the specimen in a uniformly thin deposit of stain. Resolution of molecular features is only accomplished at the stain-specimen boundary where there is maximum contrast. This result is only achieved if the deposition of buffer salts or other materials with densities less than the stain at that boundary is severely limited; otherwise the specimen molecules will be imaged at low resolution and will appear as nondescript blobs.

The specimen sample is usually applied directly to the surface of the support film where a population of specimen particles becomes adsorbed. Attachment of the molecules is usually secure enough that they are not removed by subsequent rinsing and staining operations that do remove most of the buffer salts. Since different specimens often have different affinities for the particular support film being used, some measure of control over how much specimen attaches to the film may be effected by adjustment of the specimen and buffer concentrations, adsorption time, etc. Appropriate conditions must be established by experiment for each new specimen. For protein solutions, typical concentrations range between 50-500 μg/ml and adsorption times from as little as a few seconds to several minutes. Stains are usually applied in a range of concentration from 0.25-4.0%. Adjustment of stain concentration provides some control over the thickness of the deposit.

It does **NOT** follow that a procedure that is successful with one type of specimen is also suitable for another; so various modifications should always be tried until good contrast and spreading conditions are achieved.

There are two common procedures for preparing negatively stained specimens on EM grids:

Adhesion (drop) method (Figs. II.41 and II.42)

A droplet of specimen is placed on the surface of the grid support film, making sure it sufficiently wets the surface. After an appropriate time interval, excess specimen is wicked away by touching a piece of filter paper to the edge of the grid surface. Without letting the grid dry, a droplet of rinse or stain solution is applied to the grid. Rinsing is necessary if the specimen preparation contains high concentrations of buffer salts or other solutes that may interfere with deposition of stain. The nature of an appropriate rinse depends on the conditions that the specimen can tolerate. Many viruses, for example, can withstand rinsing with distilled water. In some instances the stain solution can itself act as a suitable rinse. After rinsing and staining, excess fluid is wicked from the grid, leaving a thin aqueous film on the surface, which is left to dry, usually in air.

The specimen can also be applied to the support film by floating the grid on top of a droplet of the specimen solution. The grid is then transferred to droplets of rinse and stain solutions and then dried as before.

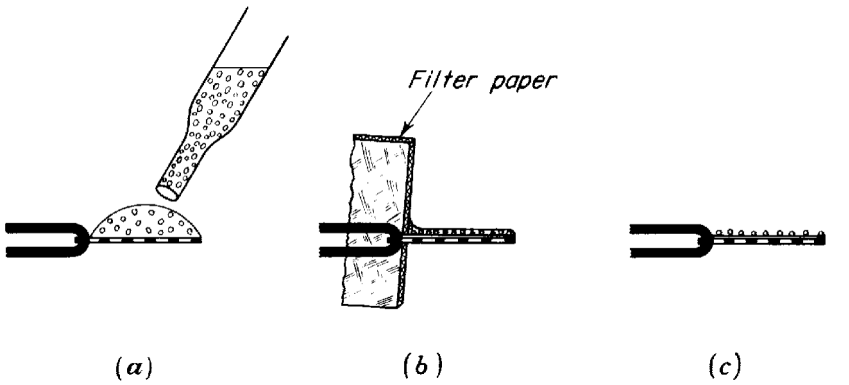


Fig. II.41. Preparation of a specimen from particles in aqueous suspension. (From Hall, 1966, p.290)

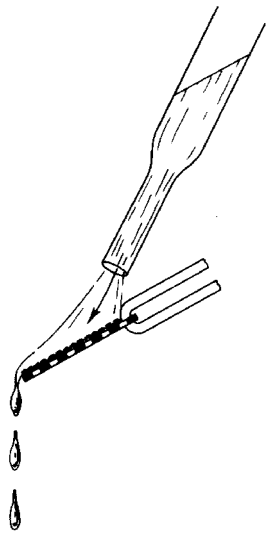


Fig. II.42. Washing a specimen. (From Hall, 1966, p.290)

An additional variation of the usual adhesion method is to apply the sample to a holey support film in the same way as is done on regular films. The sample dries in a thin layer of stain stretched out over the holes, thereby giving maximum contrast since there is no plastic and/or carbon support. Also, the stain tends to be more evenly distributed around the particle although the particle often undergoes distortions (shrinkage and flattening) due to the surface tension forces created as the layer of stain dries. The stain layer also has a tendency to break either before or after it is exposed to the electron beam. The layer of stain can be stabilized with a thin layer of evaporated carbon. Another advantage of this technique over the usual method is that, if small enough, the specimen particles will be randomly oriented in the stain layer. On regular support films, particles often settle on the surface of the film in one or a few preferred orientations, thus limiting the possible views of the specimen.

Spray droplet technique (Fig. II.43)

The normal adhesion method of preparing a particulate suspension may lead to erroneous conclusions about the relative proportion of particles since different particles are likely to have different affinities for the substrate. Also the microscopist may select fields attractive to the eye but which are not representative. The only reliable way of preparing specimens without introducing a bias is to dry a drop of the original sample in its entirety. Non-volatile salts and buffers must be removed by centrifugation and washing or by dialysis so they don't obscure the particles under study or alter the structure when the salt concentrates in the last stages of drying. The entire residue from the drop must be examined, thus it is necessary to obtain very small drops. The suspension is atomized as a fine mist and the droplets are allowed to impinge on the substrate.

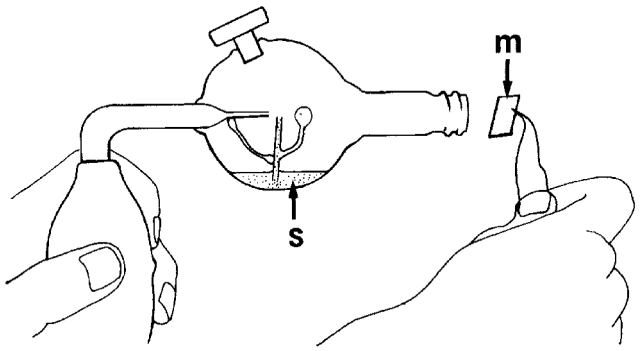


Fig. II.43 A simple hand-held nebulizer from which the sample (s) is sprayed onto mica (m). (From Willison and Rowe, 1980, p.69)

The spray droplet technique is particularly useful for examining specimens that adsorb so poorly to the support film that application and removal of rinsing and stain solutions also removes the specimen. Appropriate volumes of the specimen and stain solutions are mixed and sprayed in small droplets onto a wetable support surface. If the solution itself has the propensity to wet and spread over the surface, uniformly thin deposits of negatively stained specimen result. The resultant aqueous film will be of uniform depth, and the mass of stain deposited per unit area of support film tends to be constant. When the specimen appears to have dried, some water may still be present in the stain bed, and a rearrangement of the stain deposit could result from its rapid vaporization if the specimen is suddenly placed in the vacuum of the microscope. Thus, the specimen is usually allowed to dry (sometimes over a desiccant) for at least 10 minutes.

Drying of the aqueous film proceeds from the edges, the central area covered by the droplet being last to dry. Minor solutes tend to be held in solution until the last stages of drying and are deposited in highest concentrations in the central area. As a result, the specimen in this area ordinarily is of inferior quality.

Note that, using the adhesion drop method, there may be preferential adherence of particles so relative particle distribution counts cannot be made. A major advantage of the spray technique over the adhesion method is that preferential adherence to the grid of one type of particle over another type of particle in a mixture cannot occur. Thus, this is the method of choice in quantitative studies where relative concentrations of particles in the sample need to be determined. By knowing the volume of the original drop (from adding known concentrations of polystyrene spheres) a count of the number of particles in a drop pattern provides immediately the number of particles per unit volume (Fig. II.44: Note that this figure shows a metal-shadowed specimen to illustrate the spray droplet technique). This, together with the mass per unit volume obtained by

weighing the dried residue from a measured volume can be used to calculate a value for the mean molecular weight of the particles.

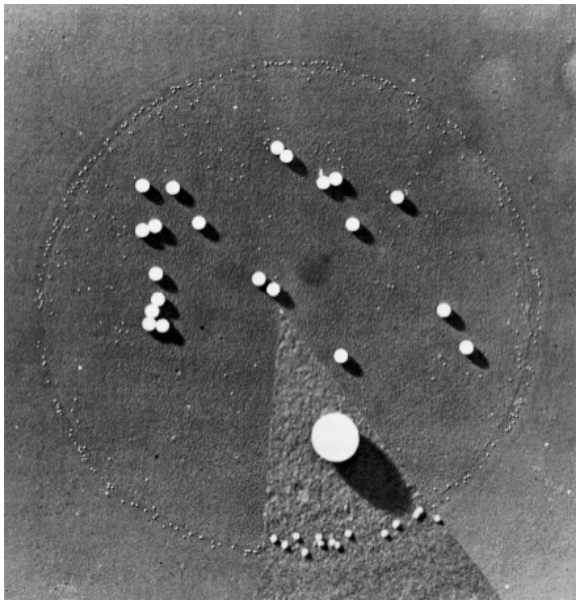


Fig. II.44. Drop pattern observed by metal shadowing. The small particles are tomato bushy stunt virus, and the large spheres are polystyrene latex particles 2,600 Å in diameter. The wedge-shaped sector has been magnified and superimposed. (From Hall, 1966, p.359)

II.A.4. Metal Shadowing

a. Introduction

The metal shadowing technique, first introduced by Williams and Wyckoff in 1945, involves the evaporation of a thin layer of metal in a vacuum onto the specimen from one side (Fig. II.45). The thickness of the metal that the imaging electron beam passes through on subsequent examination varies from place to place on the specimen depending on the local surface structure. On the side of objects away from the source of metal there will be little or no metal on the background film: these areas will appear as dark shadows on the electron micrograph since more electrons will fall in that part of the image than from the other side where the metal is thickest. Thus, the metal atoms coat the surface features of the particles, only revealing the surface details of the specimen, and also leave electron transparent areas or "shadows" on the substrate film. The overall effect, when suitably printed (*i.e.* with reverse contrast), is a lifelike effect of light and shadow, immediately interpreted by the eye in topographical terms, showing the shape and surface features of the particles very clearly.

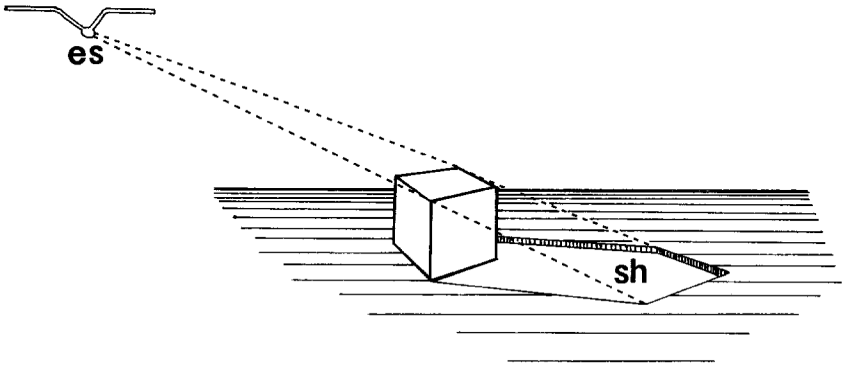


Fig. II.45. In metal shadowing, the vaporization of metal from an evaporation source (es) produces a shadow (sh) of an intermediate object, from which the metal is excluded. (From Willison and Rowe, 1980, p.5)

The resolution of the technique is generally much poorer than negative staining because the evaporated metals tend to deposit in coarse, granular particles and not as an atomic matrix (Fig. II.46). Shadowing is rarely used with sectioned material since the embedding material would have to be removed to be effective, but this would lead to collapse and distortion of the specimen.

The increase in (aperture / scattering) contrast is high using the metal evaporation technique and some indication of how thick objects are can be obtained from the shadow lengths (see § II.A.4.d). A layer as small as 2 nm (*e.g.* of platinum) gives high scattering contrast.

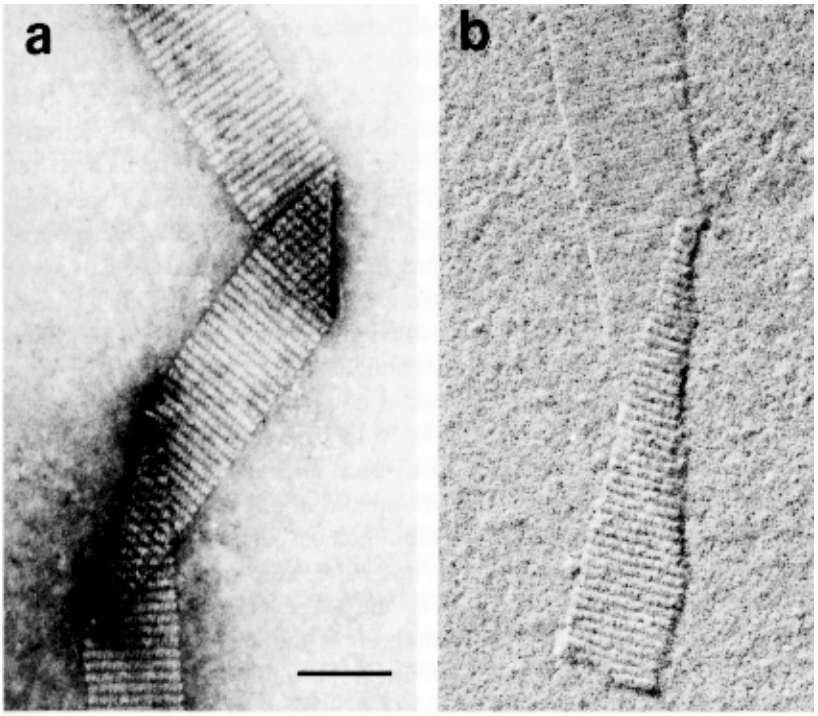


Fig. II.46. Protein structures ('spinae') separated from the surfaces of bacteria: (a) the structure negatively stained; (b) shadowed preparation showing the distinction between the relatively coarse 'upper' surface and the relatively smooth 'lower' surface. Bar = 100 nm. (From Willison and Rowe, 1980, p.61)

At high magnifications (>50,000X) specimen details start to be obscured by granularity in the metal. Some metals give finer grain than others, but the usual rule is that these are hardest to evaporate. Simultaneous evaporation of carbon and platinum (using special rods of fused carbon and platinum) gives an almost structureless film. Platinum and uranium are harder to evaporate but are frequently used in more refined, high-resolution work. Palladium is relatively easy to evaporate and has reasonably fine grain.

b. Vacuum evaporation

The apparatus used to evaporate metal atoms is the same as that used to evaporate carbon in the formation of carbon support films (Figs. II.8 and II.47). It consists of a bell jar evacuated by a diffusion pump that is backed by a mechanical pump. Inside the vacuum chamber is a base plate with vacuum and electrical feed-throughs. Substances are usually evaporated from a heater filament usually made of tungsten (Figs. II.45 and II.48). Atoms vaporized from the filament travel in straight lines and deposit as thin films on any surface in their path. The mechanical rotary and oil diffusion pumps are used in series to produce a vacuum of about 10^{-5} Torr, which is low enough so the mean free path of vaporized atoms is long compared with the distance they must travel. Most substances to be vaporized can be evaporated from filaments formed of tungsten wire of 15-30 mils diameter. The

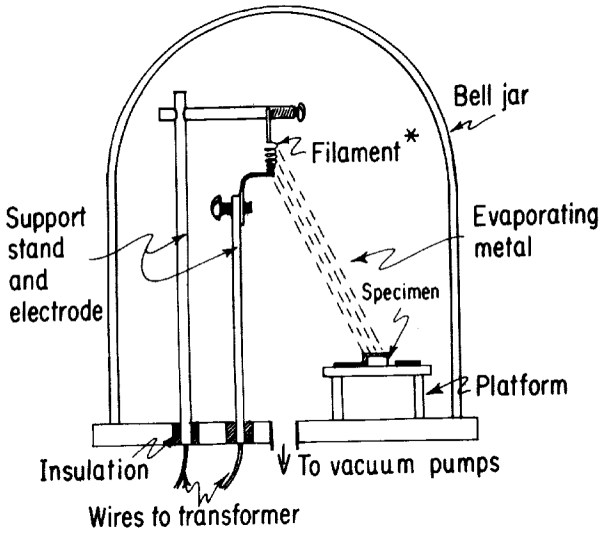


Fig. II.47. Apparatus and mechanism used to perform shadow casting. *Tungsten filament coated with melted metal (*e.g.*, platinum). (From Wischnitzer, 1970, p.244)

electrical system is a low voltage (6-12 V), high amperage (30-50 amp) type.

c. Shadowing metals

The chief requirements of a shadowing metal are chemical and thermal inertness, high density to provide maximum electron scattering for minimal deposit thickness, the capacity to be deposited in a uniform almost structureless form and to resist recrystallization and creep of deposit (which would change the apparent specimen structure).

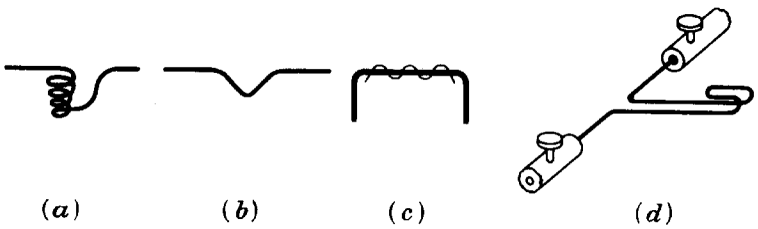


Fig. II.48. Filament types for vacuum evaporation. (From Hall, 1966, p.281)

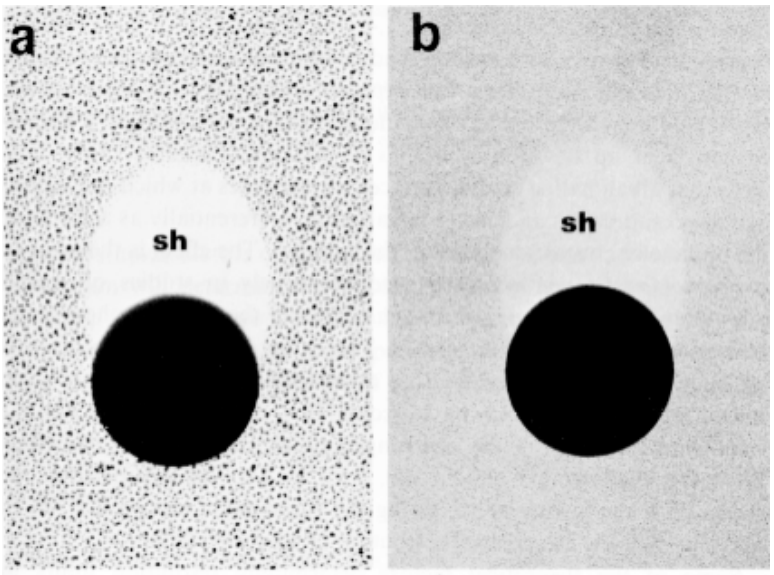


Fig. II.49. 0.31 μm diameter latex spheres shadowed with: (a) gold; (b) gold/palladium alloy (60:40). Shadow (sh). (From Willison and Rowe, 1980, p.9)

Metals for shadow casting should not show a granularity comparable in magnitude with the structural surface details to be studied and should not migrate over the surface. The density of material is of prime consideration. Some of the more commonly used metals include gold, palladium, chromium, nickel, germanium, platinum and uranium (Fig. II.49). Chromium provides films of thickness down to about 5 nm that adhere tightly to substrates but may appear granular in the TEM. Because of its low density, Cr must be deposited in thicker layers than other metals. Platinum (used as Pt-Pd or Pt-C mixtures) or uranium (as uranium oxide) provides useful deposits as thin as 0.3-0.5 nm. Platinum can be troublesome to evaporate since it alloys with tungsten and may melt through the filament, causing a break before evaporation occurs. Pt-Pd (3:1) is easier to evaporate than pure Pt. Pd melts first, alloying with the Pt and apparently protects the tungsten wire from attack by Pt.

The calculated thickness of films deposited in the plane of the specimen may vary from 0.3-1.5 nm for the heavier metals Pd, Pt and U and from 1-5 nm for Cr. Even though the metallic vapor is at a high temperature, the amount of metal deposited on a specimen is so small that no apparent damage to the specimen results. Thermal radiation from the evaporation filament can be a more serious source of damage to specimens.

In most applications of vacuum evaporation the amounts evaporated are 5 mg or less. A rough estimate of the amount of material deposited as a film on a surface normal to a line drawn to the source is given by:

$$w = \frac{M}{4\pi R^2}$$

where R = distance from source
M = total mass evaporated
w = mass per unit area deposited

This is based on the assumption that the material is radiated symmetrically in all directions. This assumption may not always be accurate.

d. Shadow casting

The method consists essentially of evaporating a metal from a source at an oblique angle to the specimen (Figs. II.45 and II.50). If the specimen is not flat, metal piles up on the surfaces that face the source, but surfaces facing away from the source are shielded and receive no deposit. Thus, relatively higher portions of the specimen cast partial or total shadows in the direction away from the source. The areas coated with metal scatter electrons most while the "shadows" scatter few or no electrons. Thus, in photographic negatives (*i.e.* the electron micrograph itself) the shadows appear black whereas metalized surfaces are light.

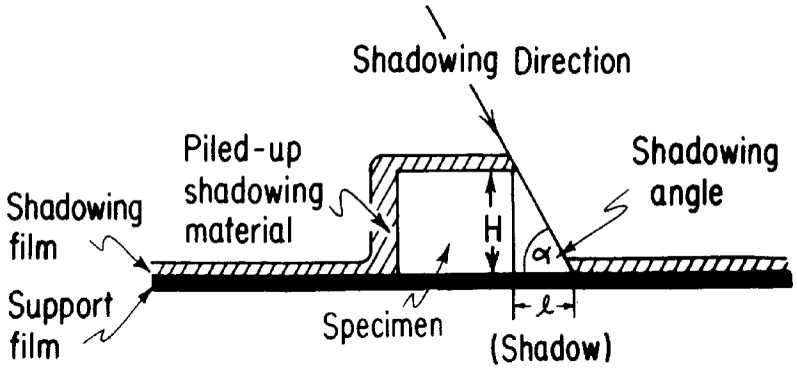


Fig. II.50. Enlarged representation of the shadowed specimen area shown in Fig. II.47. (From Wischnitzer, 1970, p.244)

Shadow lengths are used to estimate particle heights. The height of the specimen area casting a shadow can be calculated from the equation:

$$h = l \tan \theta$$

where h = height of feature casting the shadow
θ = angle of shadowing
l = length of shadow

In general, for large objects, a shadowing angle of about 45° is desirable, whereas for small or spherical objects, angles as low as 5-10° may be needed.

Uncertainty in calculations of thickness may arise from differences in the density of bulk metal versus evaporated metal, from incorrect determination of the shadowing angle (for example, if the particle is on a substrate film which is not horizontal), and from particles that are partially sunk in the substrate film. If the coating is too heavy, fine structures will be obscured. If the angle is too shallow, there will be loss of detail behind projecting features of the specimen.

To measure heights, the shadow casts should be sharply bounded. This requires a small evaporation source and good vacuum. Diffuse shadows result from poor vacuum. Shadow casting is effective in increasing aperture contrast of small particles, but what is seen in the image is a metal cap, which exaggerates the dimensions of small objects. The apparent size of objects also depends on their relative orientation to the metal source. For example, the width of tobacco mosaic virus rods measured from shadowed preparations depends on the orientation of the rods with respect to the shadow direction. Rods aligned nearly parallel to the direction of shadowing gives the most accurate value, whereas rods oriented perpendicular to the shadow direction will give incorrect width measurements. Additional uncertainties arise because of dehydration-induced collapse or distortion of the structure.

e. Rotary shadowing

Continuous rotation of the object during deposition of the metal causes measurable shadows to be lost, but the buildup of metal on projecting objects is uniformly independent of their azimuth and details of structure, which might be lost because they are not favorably oriented or are located in the shadow of other objects will be metalized.

f. Surface replicas

Thick specimens (>0.1 μm) cannot be studied directly with the electron microscope because too much of the beam is scattered and absorbed. The surface detail of thick specimens is investigated by casting a replica of them (Figs. II.51 and II.52). The specimen is then dissolved away leaving only a thin replica to be viewed in the microscope. Replicas are also useful for the examination of materials, which are too volatile or unstable to be placed in a vacuum or exposed to the electron beam.

Replica methods all require an initial impression of the surface to be produced. Replicas consist of thin films of electron-transparent and electron-dense material, usually metal-coated carbon or Formvar, which corresponds exactly to the surface topography of the specimen. Since the replicas themselves are very thin (~20 nm) they have very little contrast of their own and must be shadowed with evaporated metal. A major difficulty with the technique is separating the replica from the surface to be studied. In general, this necessitates the destruction of the specimen by dissolving it away from the replica. In some cases it is possible to separate the two simply by flotation.

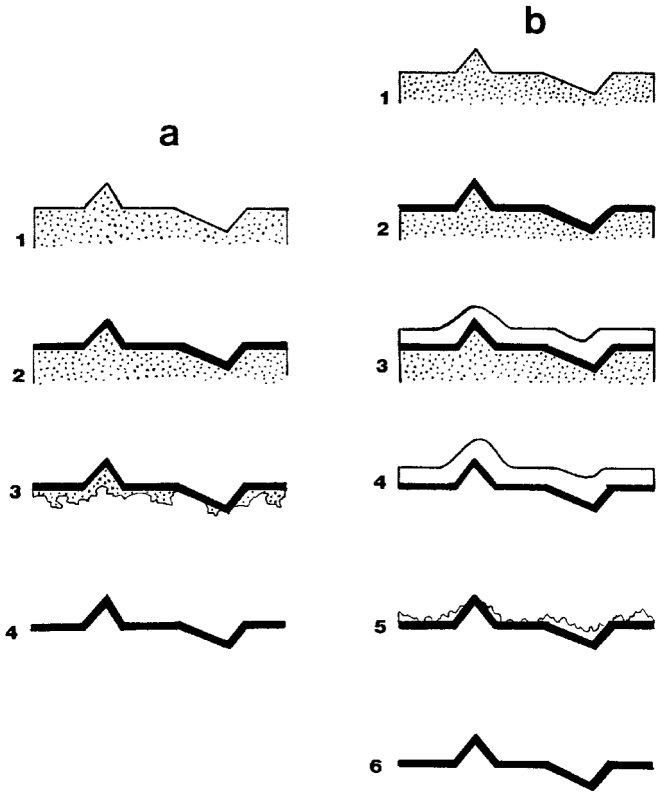


Fig. II.51 Two methods of separating replicas from surfaces. (a) By dissolution of the specimen: (1) the specimen; (2) replication; (3) dissolution of the specimen; (4) the replica. (b) By stripping using a backing film: (1) the specimen; (2) replication; (3) application of the backing film; (4) the replica stripped from the specimen with the assistance of the backing film; (5) dissolution of the backing film; (6) the replica. (From Willison and Rowe, 1980, p.99)

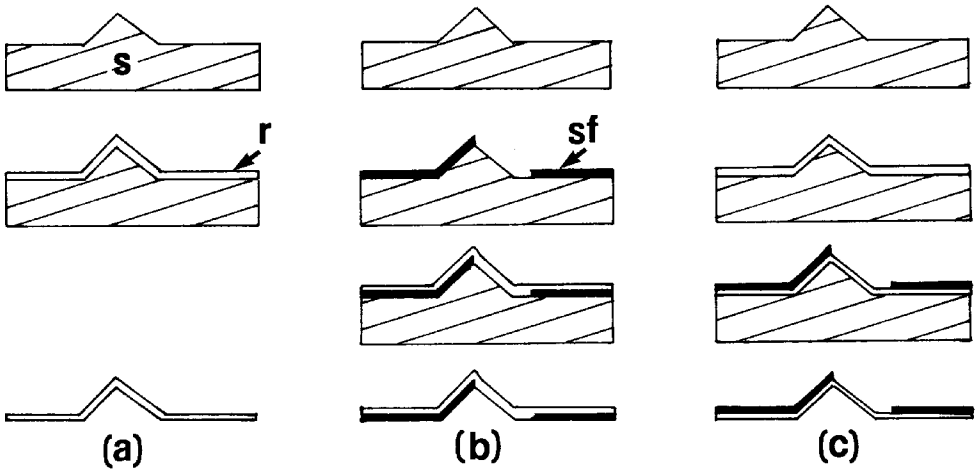


Fig. II.52 Single-stage replicas: (a) an unshadowed replica; (b) a pre-shadowed replica; (c) a post-shadowed replica. Specimen (s); replica (r); shadowing film (sf). (From Willison and Rowe, 1980, p.6)

There are two basic techniques for preparing surface replicas:

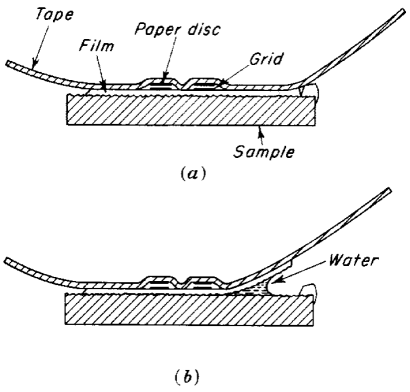
1) Negative replicas (Figs. II.53 and II.54)

The simplest method of replication, also known as the single-stage replica method, provides a negative replica, so called because heights and depressions are reversed with respect to the original

surface. The three steps generally involved are:

- Apply a solution of plastic or resin to the surface and let dry
- Separate the film from the surface
- Shadow the replica

The main difficulties encountered are in the stripping operation. Thick films from concentrated solutions or smooth surfaces strip most easily.



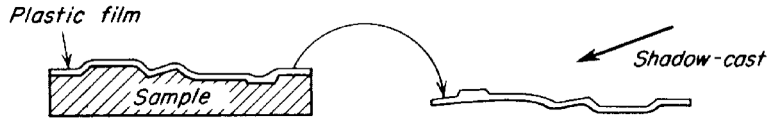


Fig. II.53. Negative-replica technique. (From Hall, 1966, p.310)

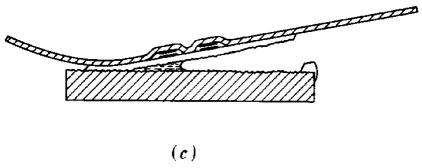


Fig. II.54. Wet stripping of a negative-replica . (From Hall, 1966, p.311)

2) Positive replicas

This requires an intermediate negative replica from which the final positive replica is made. A preliminary impression of the specimen surface is made with one material and then this is separated from the specimen and coated with the replicating material (Fig. II.55). The primary cast of the surface (negative replica) is made with a thick layer of plastic, which can then be stripped off the surface simply by pulling. Since the negative replica need not be thin, it can be separated from the sample by force if necessary. The underside of the primary replica, bearing the imprint of the specimen surface, is coated with the second replicating material and then the plastic in the intermediate replica is dissolved away, leaving the secondary replica intact. After shadowing, the final positive shows heights and depressions as they are on the original sample. A number of pairs of substances have been found satisfactory for the method, for example, carboxymethyl cellulose in water can be used to make an intermediate negative replica and the final positive replica is then made with collodion in amyl acetate.

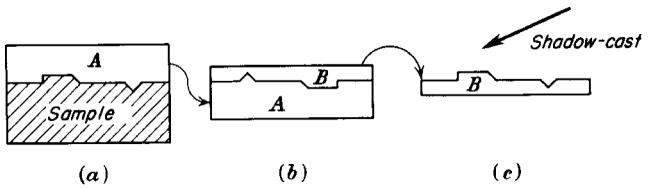


Fig. II.55. Positive-replica technique. (From Hall, 1966, p.314)

g. Shadow transfer techniques

The surface to be examined is first shadowed and a collodion film is used to strip and transfer the metal deposit. The resultant specimen is called a preshadowed replica or a pseudo replica. Surface irregularities of the substrate must be considerably smaller than the dimensions of the particles to be examined. Such methods are classified as transfer techniques because, although they do give a picture of surface contours, like replicas, the picture is like that of any shadow cast specimen. The structure seen is precisely what would be seen if the original material were thin enough to be viewed by transmission microscopy after shadow casting. Essentially the method consists in transferring the metallic deposit from the original surface to a thinner support.

Glass seems to be a good substrate, even better than mica or polished metal. Pt-Pd alloy seems to be the best metal for transfer from glass. 1% ethyl cellulose in ethylene chloride seems to be better than collodion surfaces.

Mica substrate technique

Mica is good because it doesn't have irregularities on the order of the particle dimensions. It is also chemically inert and hydrophilic. Since it can't be easily split to thicknesses that can be used

directly in the TEM, its use entails a stripping or replica technique. It is usually best to suspend materials that are to be deposited in volatile salts and buffers and apply them with the spray technique (Fig. II.56).

Advantages of shadow transfer over replication for examination of surfaces:

- The original surface structure is shadowed.
- No pressure or heat is required.
- The micrograph shows heights and depressions as they are in the original.

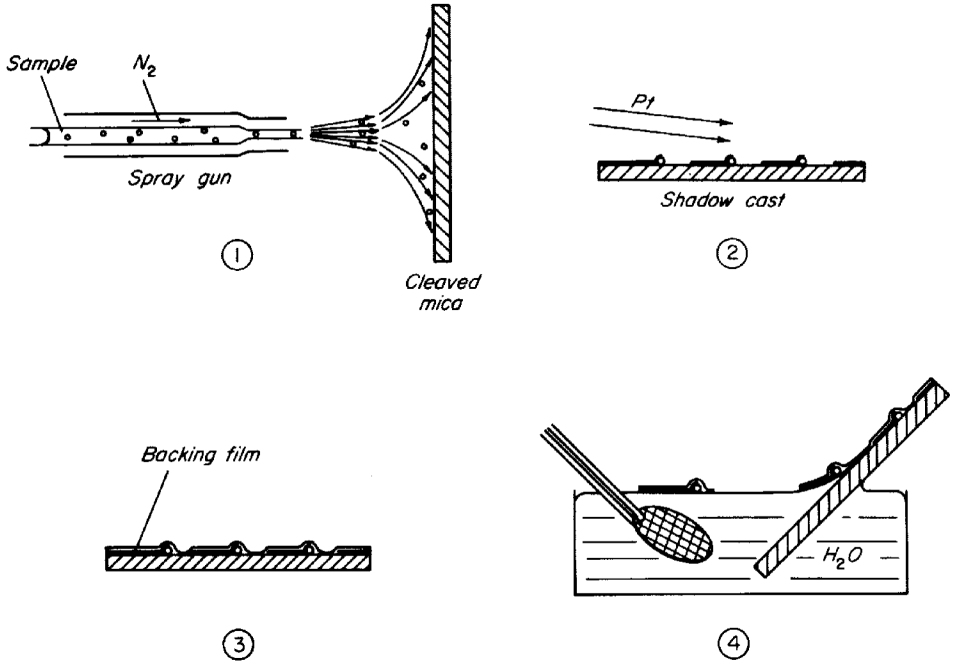


Fig. II.56. Mica technique for observing macromolecules. (From Hall, 1966, p.323)

h. Surface spreading technique

This technique is most useful for examining DNA and RNA molecules. A shallow, rectangular glass tray is filled with a supporting liquid (distilled water or 0.15M ammonium acetate). The nucleic acid-containing specimen is suspended in a protein-salt solution (*e.g.* 1% cytochrome c in 1M ammonium acetate). After cleaning the surface of the liquid by placing two Teflon bars side by side across the width of the trough and slowly drawing them apart, talc is spread on the surface to serve as a marker for the spreading film. The protein-nucleic acid solution is applied slowly to the surface of the water. The protein immediately spreads out in a monolayer leaving a clear area in the talc (Fig. II.57). The monolayer is then mounted on carbon-coated support grids and positively-stained with uranyl salts or rotary-shadowed with metal at a low angle. Rotary shadowing causes metal to impinge from all directions and avoids the difficulty that segments of molecules tend to become invisible when they are in line with the shadow direction (Figs. II.58 and II.59).

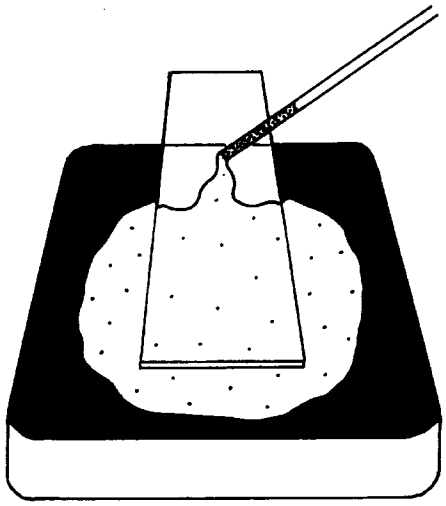


Fig. II.57. Use of a dish for spreading nucleic acids. The DNA-cytochrome c solution (stippled region) is applied from a microcapillary tube down a glass slide. It spreads across the hypophase solution, pushing away the talc (dark area) sprinkled on the surface. (From Sommerville and Scheer, 1987, p.9)

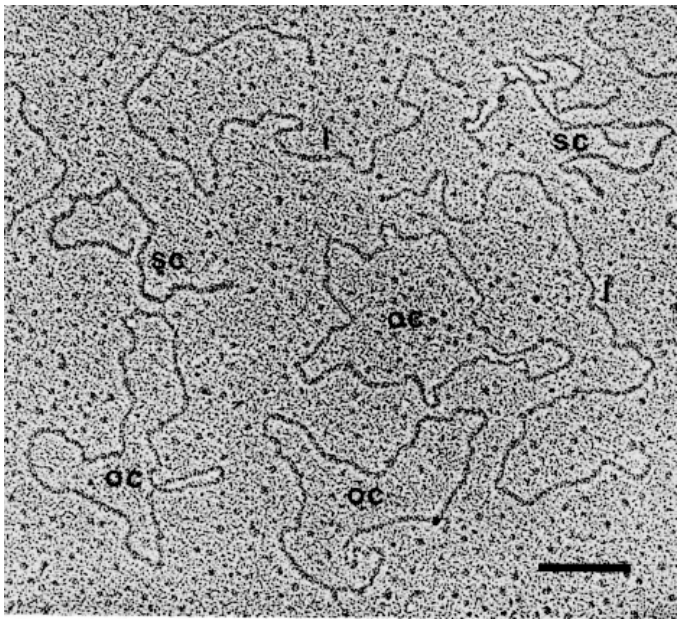


Fig. II.58. ColE1-derived plasmid DNA, spread in ammonium acetate. Super-coiled (sc), open circular (oc) and linear (cut with *Eco*RI restriction endonuclease) (1) forms of DNA, are present. Bar represents 0.25 μm. (From Sommerville and Scheer, 1987, p.12)

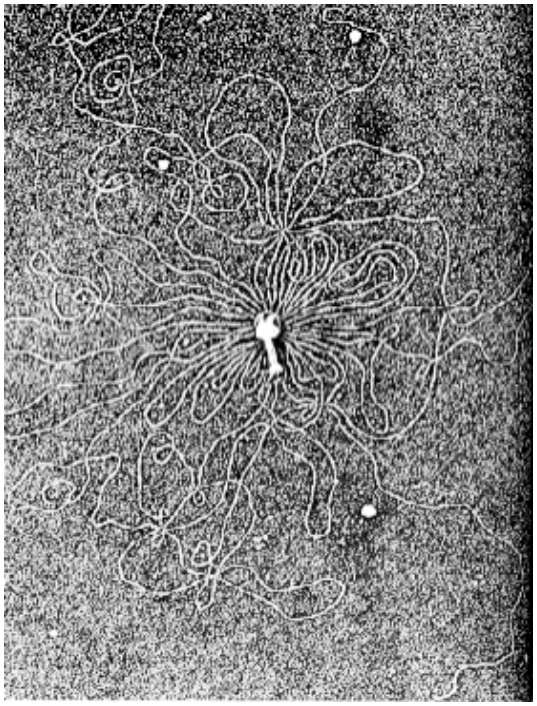


Fig. II.59. Demonstration of the surface spreading technique. An osmotically shocked T2 bacteriophage who's DNA content has been spread out as fine strands from the core of the virus particle. (From Wischnitzer, 1981, p.206)

II.A.5. Freeze-Drying/Fracture/Etching

a. Freeze drying (Figs. II.60-II.62)

Freeze drying of biological specimens is a means for avoiding distortions of structure associated with the removal of water. Specimen samples are prepared by the adsorption technique and just before the liquid film dries, the grid is plunged into liquid nitrogen (or, better yet, into a cryoprotectant such as isopentane or ethane cooled to liquid nitrogen temperature). The frozen material is held at low temperature (~ -100 to -80°C) in a vacuum until the water leaves by sublimation. The specimen is relatively rigid in the frozen state, thus forces due to surface tension are reduced or eliminated.

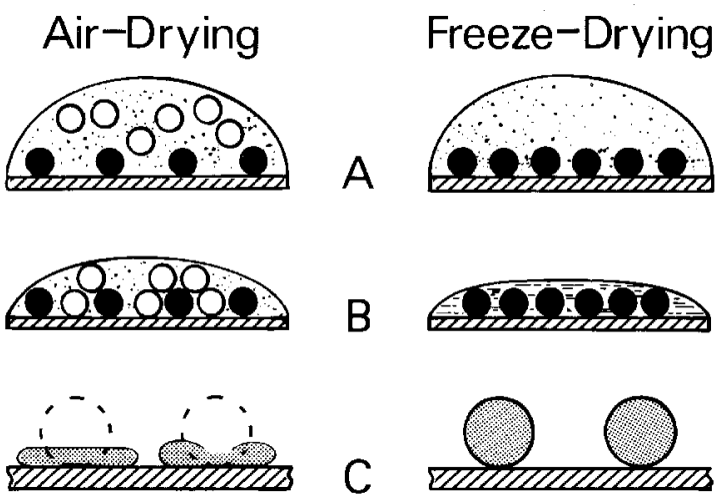


Fig. II.60. Sequence of events during conventional air-drying and during freeze-drying on a grid. (A) Both adsorbed and unadsorbed particles are present in the capillary layer of liquid before air-drying. A monolayer of adsorbed particles is present on the grid as prepared for freeze-drying. (B) During air-drying the unadsorbed particles aggregate or overlap; this is not the case in freeze-drying. (C) During air-drying, surface tension forces cause collapse or disruption of particles. The three-dimensional structure of particles is well preserved after freeze-drying. (From Nermut, 1977, p.80)

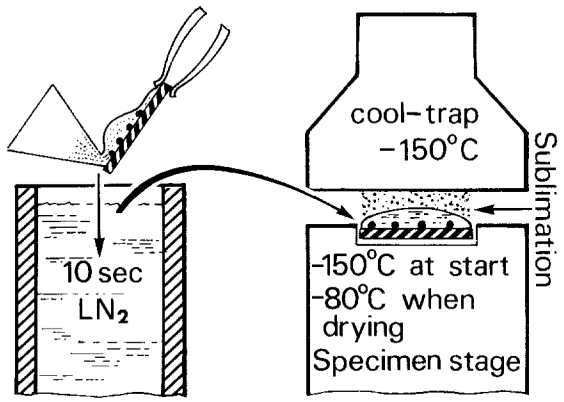


Fig. II.61. The freeze-drying procedure. After the last wash the excess liquid is drained off with a filter paper and the grid is dipped quickly into liquid nitrogen. Then it is rapidly transferred onto the precooled specimen stage of a freeze-etch unit and dried in a vacuum. (From Nermut, 1977, p.87)

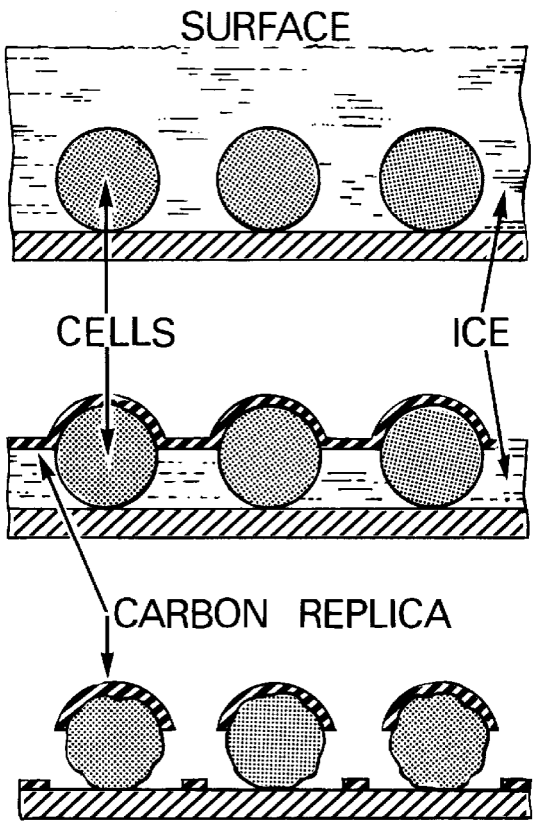


Fig. II.62. Freeze-drying of cells. Drying should be carried out as long as necessary (for 1-2 hours) to uncover the upper half of cells. The replica is more continuous than when all ice is sublimed off, and a possibility of shrink-age of cells is fairly reduced. (From Nermut, 1977, p.89)

b. Freeze-fracturing, freeze-etching

This allows investigation of objects in the frozen state. There is no chemical treatment of the object during the entire procedure until the replica is formed on an "etched", fractured surface of the frozen specimen. The resolving power of the method is limited by the coarseness of the granular deposits of evaporated metal.

The method involves three main steps, all carried out in a specially designed apparatus consisting of a microtome and a freeze-drying and shadow-casting installation, all in the same vacuum evaporator (Fig. II.63).

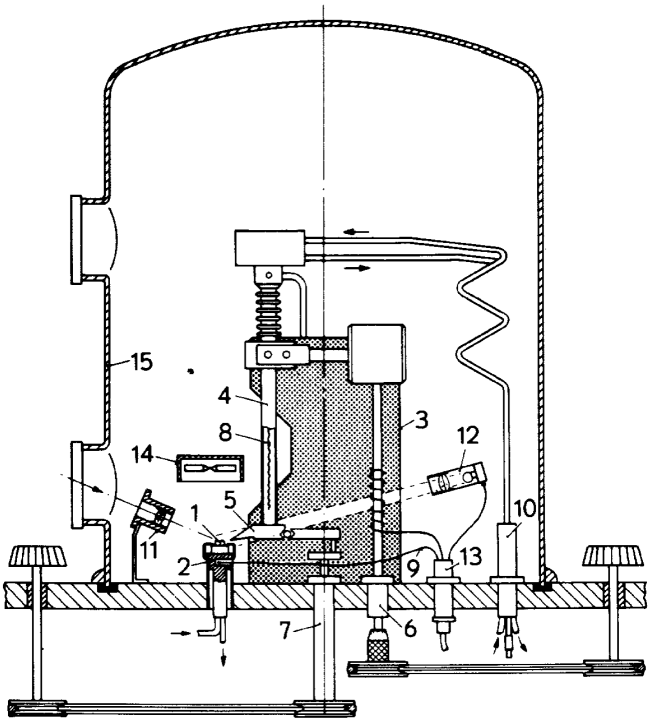


Fig. II.63. Simplified diagram of the Moor-type freeze-etch unit manufactured by Balzers AG: (1) Specimen. (2) Specimen stage. (3) Microtome stand. (4) Knife arm. (5) Knife. (6) Knife-advance mechanism. (7) Microtome drive. (8) Liquid nitrogen coolant within the knife arm. (9) Thermocouple lead. (10) Liquid nitrogen supply to the knife. (11) Magnifying lens. (12) Lamp. (13) Electrical lead-through. (14) Electrode for carbon evaporation. (15) Bell-jar. (From Willison and Rowe, 1980, p.209)

1) Freezing

A piece of tissue or cell suspension is held on a specimen support and cooled as rapidly as possible to below -100°C (Fig. II.64a,b). The specimen support bearing the frozen tissue is then introduced into a vacuum evaporator where it is rapidly evacuated and maintained at low temperature.

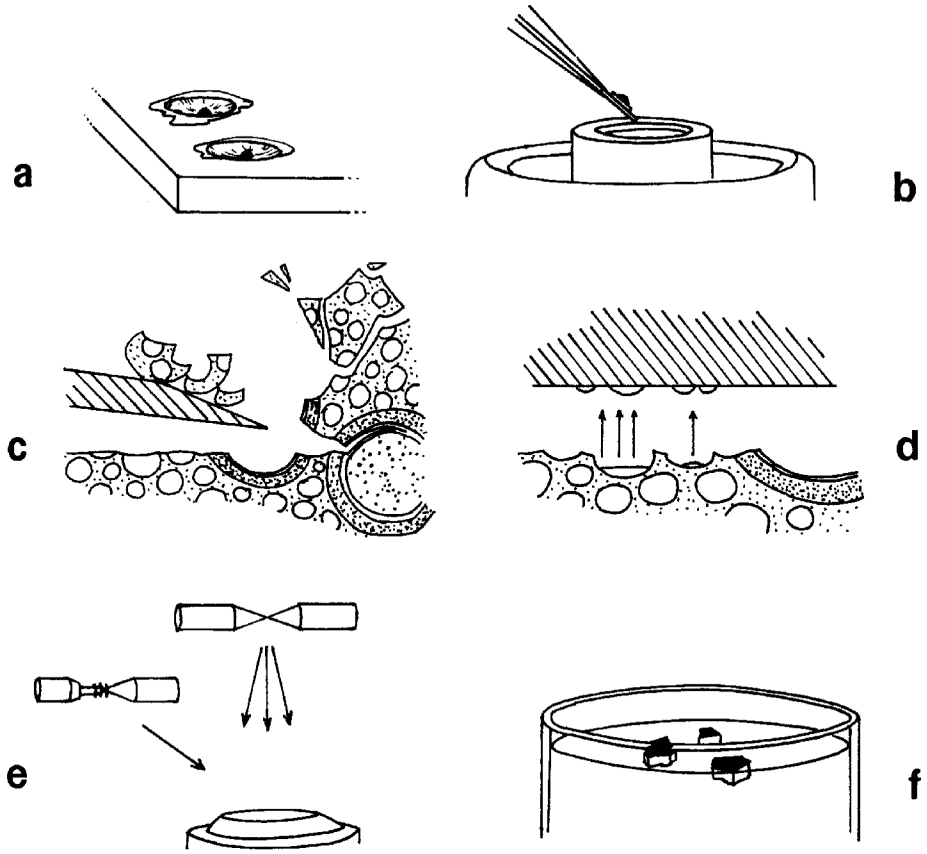


Fig. II.64. The essential steps of freeze-etching: (a) isolation and pretreatment of the specimen; (b) freezing; (c) fracture of the frozen specimen; (d) etching (vacuum sublimation of ice) in the presence of a cold-trap which prevents condensation of water vapor on the surface of the specimen; (e) shadowing and replication; (f) replica cleaning by dissolving the specimen. (From Willison and Rowe, 1980, p.173)

2) Fracturing

The frozen specimen is "chipped" (fractured) with a cooled knife, exposing a flat surface to the vacuum (Figs. II.64c and II.65b). The surface is now left for a time, during which ice sublimes (*i.e.* freeze dries at -80 to -100°C to a depth of 10-30 nm) from the cut surface, leaving the cell ultrastructure standing in relief above the ice surface. The ice sublimes onto a surface cooled with liquid nitrogen held in close proximity to the cut surface (Fig. II.64d and Fig. II.65c). After sufficient ice has sublimed, the surface is then shadowed with evaporated metal and is immediately coated with a layer of evaporated carbon (Fig. II.64e). This process leads to the formation of a shadowed replica of the cut, sublimation-etched surface.

3) Detachment

The specimen is removed from the vacuum, thawed, and the replica is removed by floating it off onto a water surface. Any adherent particles of organic matter are removed by floating the replica on a solution of caustic alkali (Fig. II.64f), after which the replica is washed with several changes of distilled water and picked up on a grid and examined in the TEM.

c. Deep-etching

This is just a prolonged freeze-etching process (Fig. II.66), which enables more of the fractured surfaces to be exposed for shadowing (Figs. II.67 and II.68).

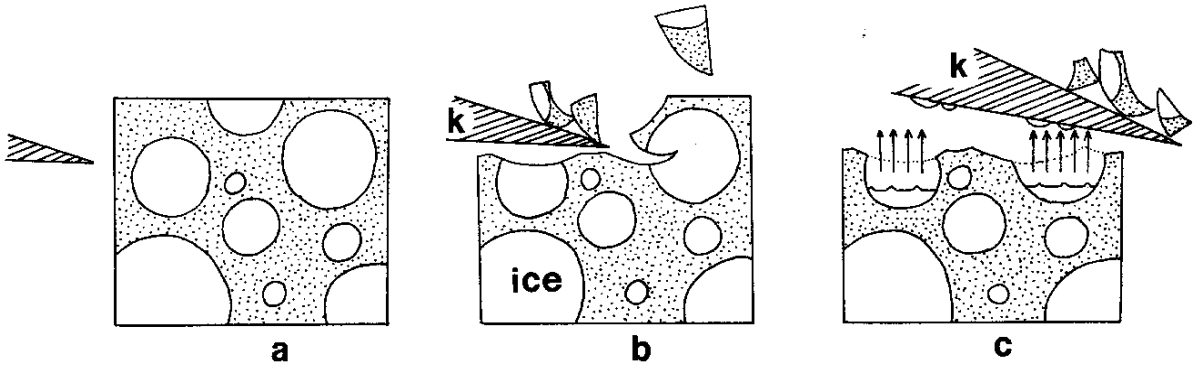


Fig. II.65. Etching. (a) When an aqueous specimen is frozen, ice crystals arise. (b) Fracturing this frozen specimen with a knife (k) exposes some ice crystals. (c) After fracturing, some of the ice sublimes from the exposed surfaces and condenses on the cold-trap (in this example, the knife). Note that the eutectic between the ice crystals does not sublime. (From Willison and Rowe, 1980, p.216)

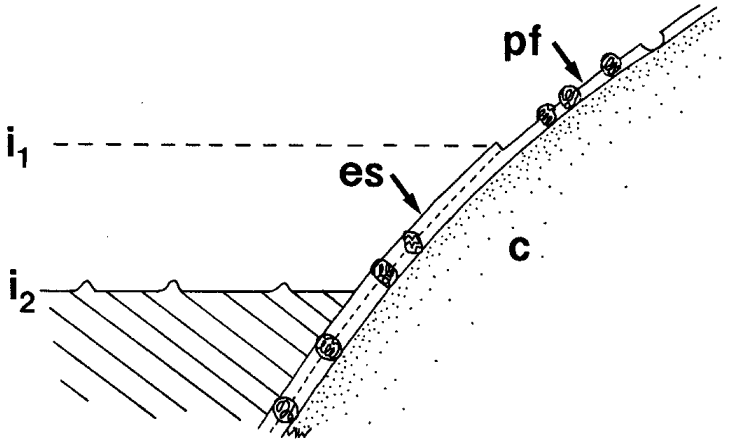


Fig. II.66 In deep-etching, the material is frozen in distilled water, fractured, and then etched at -100°C for longer than usual (*e.g.* 2 min). Thus, immediately after fracturing the ice is at i_1, but it recedes to i_2 during etching. If a biological membrane forms the ice-specimen interface, fracturing will reveal an internal fracture plane (pf) and etching will reveal the membrane surface (es). c, cytoplasm. (From Willison and Rowe, 1980, p.221)

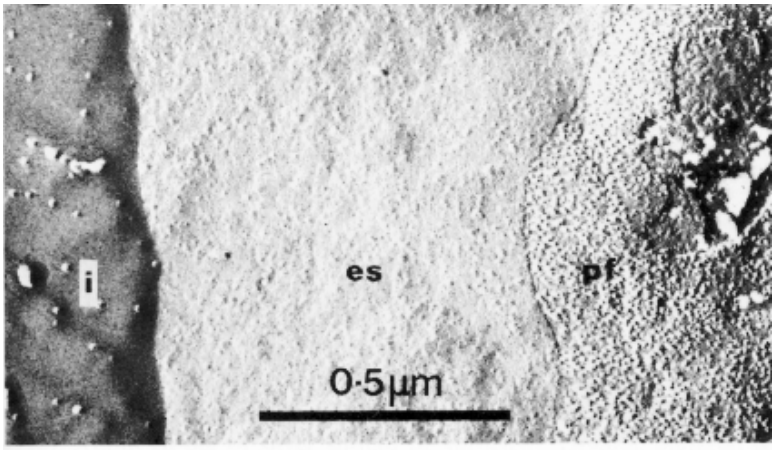


Fig. II.67 A deep-etched isolated plant protoplast, prepared as indicated in Fig. II.66. i, ice; es, outer surface of the plasma membrane; pf, P fracture face of the plasma membrane. (From Willison and Rowe, 1980, p.221)

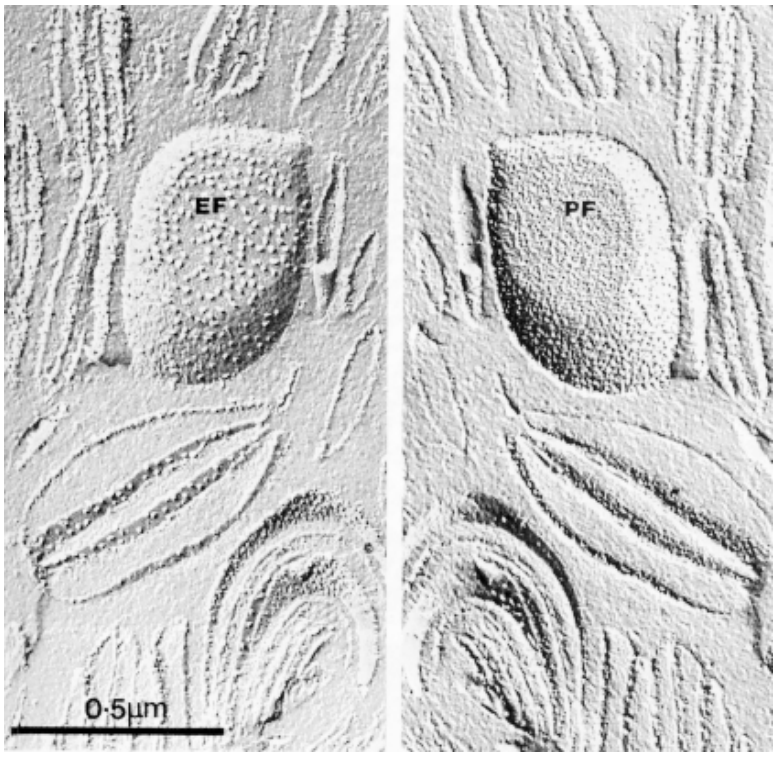


Fig. II.68. Complementary replicas of isolated chloroplast thylakoid membranes suspended in 30% glycerol in phosphate buffer (pH 7.2). The fractured specimen was replicated at -160C (no etching). Notice particularly that the membrane-associated particles of the thylakoid membranes do not segregate at random. Small holes (white specks) may be seen between the large particles of he E fracture face (EF), which complement the particles on the P fracture face (PF). (From Willison and Rowe, 1980, p.195)

II.A.6. Unstained, Vitrified (Frozen Hydrated) Specimens

The main goal in examining unstained specimens in the microscope is to overcome artifacts associated with staining and other chemical procedures and thereby attempt to record the structure of the specimen in as close to its native state as possible. Three basic procedures have been devised for preserving native biological structures in the TEM. They include direct observation of specimens that are:

- Fully hydrated (wet)
- Embedded in sugar
- Vitrified

a. Wet specimens

A number of years ago the laboratory of Don Parsons invested considerable effort in developing methodology for examining fully hydrated specimens at room temperature in the TEM (*e.g.* Parsons, 1972). A high voltage microscope was equipped with a differentially-pumped hydration stage consisting of four colinear apertures through which the electron beam passed, the specimen being positioned between two inner 75 μm apertures. A pressure gradient was maintained across the apertures by feeding a source of water vapor to the specimen and differentially pumping on the low-pressure side of the aperture. This method was deemed superior to using sealed film chambers. Electron diffraction patterns out to 0.2 nm resolution were recorded from wet microcrystals of catalase. The major drawback to this technique, and the reason it never caught on, stems mainly from the need to equip the microscope with a specially built hydration stage, thus diminishing the attractiveness of doing routine work. Wet specimens are also very sensitive to the effects of radiation damage.

b. Sugar embedding

This technique is virtually identical to the standard adsorption technique used in negative staining (§II.A.3) except that a 0.5-2% solution of sugar is used in place of the negative stain solution. The sugar provides an excellent matrix that surrounds and protects the specimen from distortions due to dehydration effects. The use of sugar embedding led to pioneering achievements in high resolution studies of crystalline specimens like purple membrane (Henderson and Unwin, 1975), which was solved to 7-Å resolution using image reconstruction techniques discussed in § III. The

major drawback to the sugar embed technique is the extremely low contrast obtained since the density of the dried sugar nearly matches the density of most biological specimens (Hence, the biological specimen becomes essentially invisible and there is little if any aperture contrast). The technique has mainly been used with specialized specimens, especially highly-ordered crystalline samples such as catalase crystals and purple membrane and cytochrome oxidase vesicles, which must be studied using image analysis techniques to enhance their inherent low contrast (§ III).

c. Preparation of vitrified specimens

This technique, though somewhat challenging to master at first, has proven to be a widely accepted way to study a variety of unstained biological specimens. The basic aim is to use physical fixation, or cryo-fixation, to rapidly freeze the biological sample so as not to disturb its aqueous environment. This, in principle, avoids ultrastructural changes, washing away of substances, and redistribution of elements. Specimens that are frozen in vitreous (*i.e.* glassy or non-crystalline) ice show a structure similar to the liquid state. If vitrification is not achieved, crystal formation and solute partition often lead to fundamental structural changes in the specimen. Thin layers of pure water or aqueous solutions can be frozen in the vitreous, hexagonal, or cubic form depending on the cryogen used and on the thickness of the layer. Vitrification is obtained with spray freezing and thin film freezing up to a thickness of ~1 μm when samples are cooled in liquid propane or ethane at about 100°K. Cubic ice crystals form when a thicker layer or a less efficient cryogen (*e.g.* nitrogen slush) is used. When freezing is performed in boiling liquid nitrogen, pure water always freezes in the hexagonal crystalline form. With solutions, the hexagonal or cubic state can also be obtained depending on the solute, its concentration, and the thickness of the layer.

Contrast in **vitrified** specimens is expected to be directly interpretable as structure rather than as stain. Artifacts associated with fixation and drying, common to other specimen preparation techniques, are avoided or eliminated.

1) Advantages of preparing vitrified specimens

- Allows the examination of "native", hydrated structural features
- Provides excellent preservation of biological structure in the microscope vacuum (*e.g.* 0.18 nm resolution for catalase crystals)
- Provides a 2-5 fold reduction in radiation damage
- Allows contrast between protein, nucleic acid, and lipid to be distinguished
- Allows one to control the chemical environment and thereby examine different functional states of molecules

2) Preparation of vitrified specimens

Techniques for preparing vitrified specimens include the following basic steps:

- Formation of a thin layer of the biological suspension
- Rapid cooling to the vitreous state
- Transfer to the EM without rewarming above the devitrification temperature (T_d = 130°K)
- Observe the specimen below T_d with an electron dose low enough to preserve the structure of the specimen

3) Techniques for preparing vitrified specimens

Three common techniques for preparing vitrified biological specimens include:

Sandwich technique

Sample is applied to one surface of a folding grid containing thin hydrophilic support films on both grids, and the grids are folded over to squeeze most of the excess liquid out (Fig. II.69). After blotting away most of the excess liquid from the folded grid sandwich with a piece of filter paper held to its edge, the sample is immediately plunged into a bath of liquid nitrogen (or other cryogen). This procedure produces sufficiently thin films of solid water or buffer within which the specimen is embedded. In some of the very earliest studies using this technique, electron diffraction of vitrified, unfixed catalase crystals sandwiched between thin, hydrophilic support films was obtained with diffraction spots out to 0.34 nm resolution (Taylor and Glaeser, 1976).

Blotting technique (Bare Grid or Perforated Film)

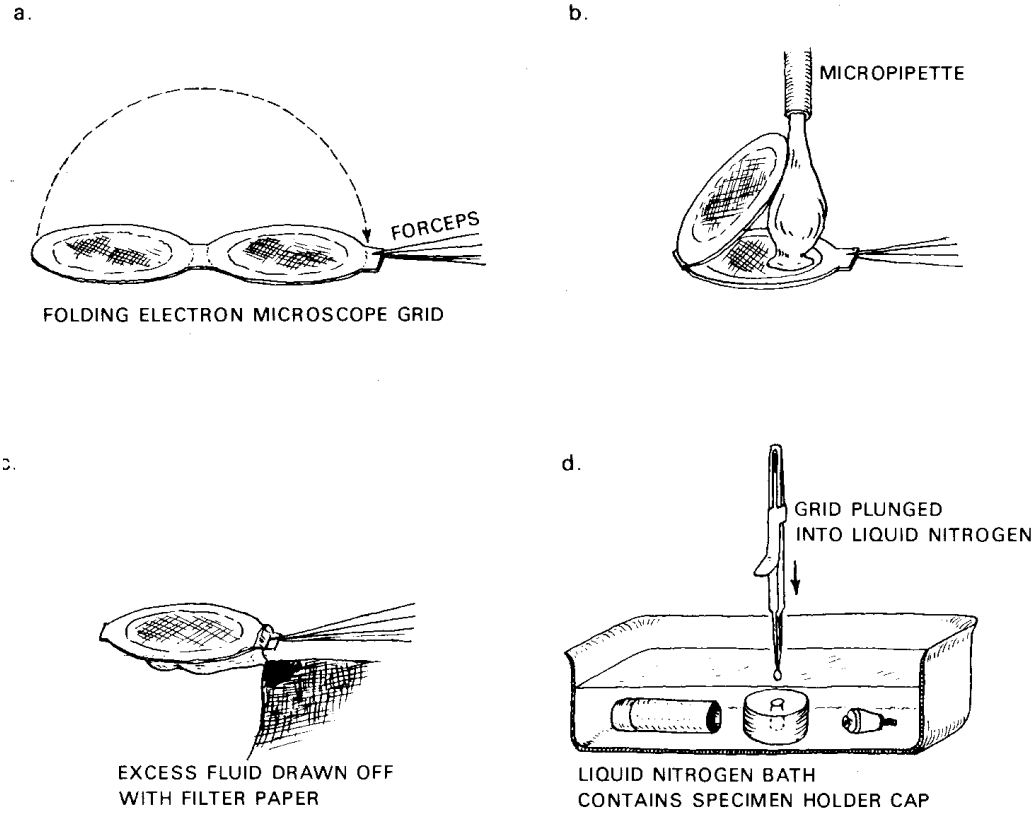


Fig. II.69. Original procedure for producing frozen thin specimens for electron microscopy. (a) A 200-mesh folding grid is partially closed. (b) A drop of suspension of specimen sufficient to fill the space between the two faces of the grid is applied. (c) The two faces of the grid are folded tightly, and the excess fluid is withdrawn with filter paper. (d) Plunging the specimen into liquid nitrogen immediately freezes it, and the grid is then placed into the specimen holder cap. The cap is mounted upright in a brass block, which is also submerged in liquid nitrogen. The cap with the specimen is mounted onto the specimen holder, which is then placed into the cold sink-frost protector for transfer into the microscope. (From Taylor and Glaeser, 1976, p.449)

A droplet of sample is applied to a strongly hydrophilic support film (or bare grid), most of the liquid is blotted away by pressing filter paper flatly against the specimen side until the liquid connection between paper and grid surface is broken, and the grid is then immediately plunged into liquid cryogen (*e.g.* ethane) at a temperature just above the freezing point of the cryogen (Fig. II.70). Only ethane or propane produce a sufficiently rapid freezing rate that guarantees formation of amorphous ice. The success of this technique depends on the ability of water to remain thinly spread on a very hydrophilic surface (often achieved by glow-discharging the grids). The blot technique is by far currently the most widely practiced method of quick freezing particulate samples of biological macromolecules. A commercial device, the Vitrobot, provides a reproducible means to obtain blotting and freezing of samples in a humidity and temperature-controlled environment (Fig. II.71).

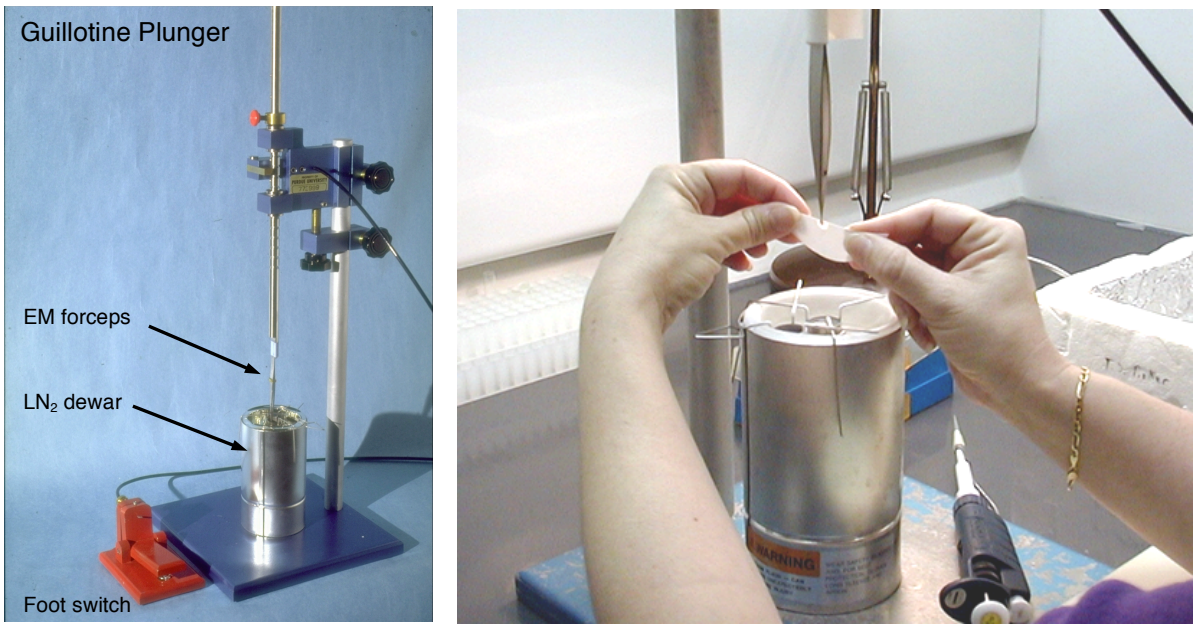


Fig. II.70. Left: Simple plunge freezing device, activated by a foot switch. Right: Blotting of sample with filter paper just before plunging it into liquid ethane cryogen. (Photos courtesy of N. Olson).

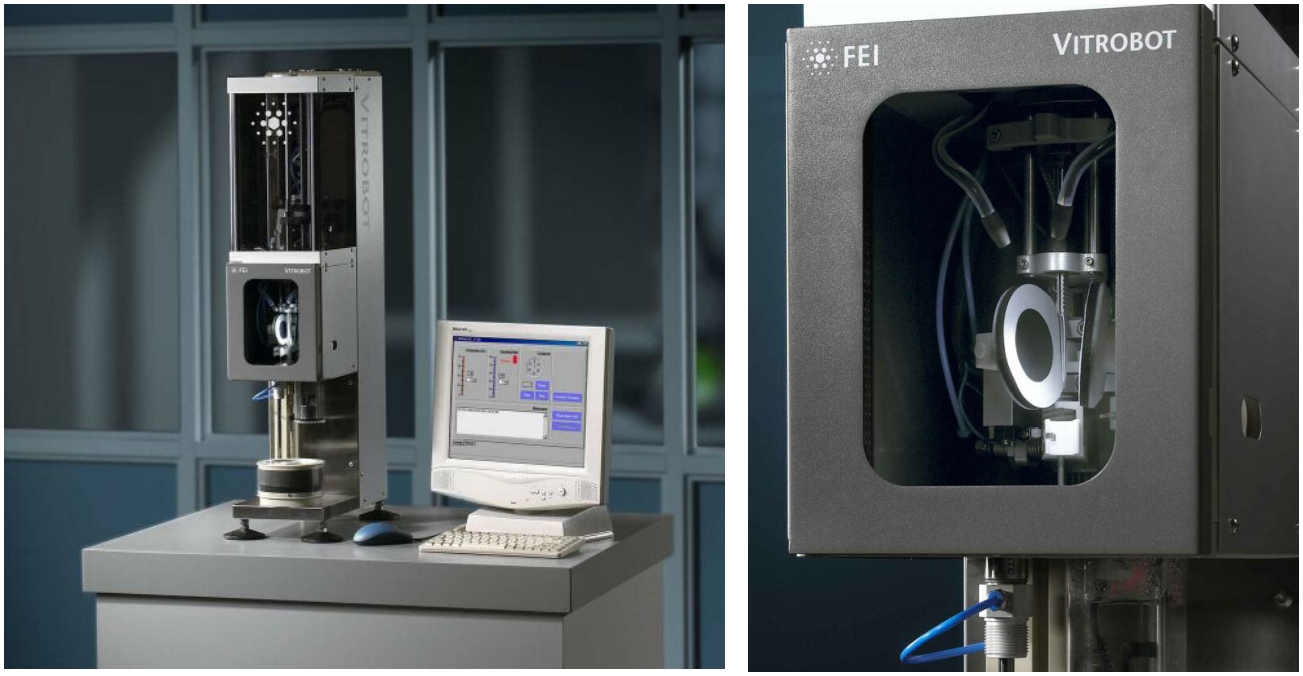


Fig. II.71. Vitrobot plunge freezing device sold by FEI Company. The device allows samples to be vitrified under controlled temperature and humidity conditions. Such control provides a means to achieve greater reproducibility in freezing experiments. (From FEI Brochure)

Spray technique

This technique is essentially the same as the blotting technique except that the sample is sprayed onto the support film while it is simultaneously dropped into cryogen (Fig. II.72).

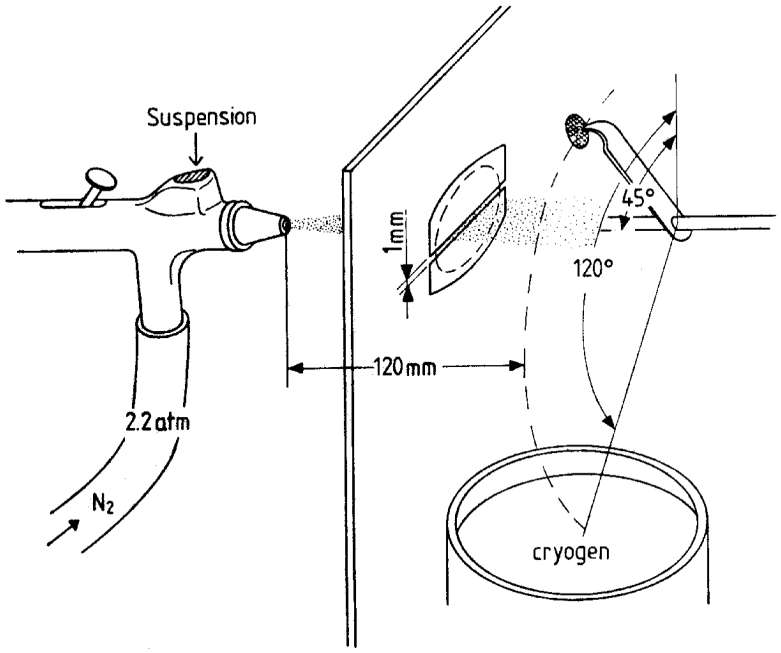


Fig. II.74. Schematic of spray freezing apparatus. (From Dubochet *et al.*, 1982, p.224)

4) Properties of frozen, aqueous solutions and preservation of biological structure

Success in not disturbing fragile biological specimens when they are frozen is apparently achieved by cooling fast enough to **vitrify** rather than crystallize the water. For example, if fully embedded in a layer of vitrified water ('amorphous ice'), catalase crystals will have their crystalline order preserved to at least 0.2 nm resolution. If the catalase crystals are then freeze-dried, order is lost to about 1 nm resolution. The crystal shrinks and lattice parameters change and cracks appear. When vitrification is not achieved, biological samples separate into domains of either pure crystalline water, or biological material and concentrated solute with some residual water.

Good preservation can sometimes even be obtained when the freezing speed is **NOT** sufficient to vitrify the bulk water. Susceptibility to ice damage varies for different specimens. Those specimens with the most openly packed structures seem to be the most sensitive to disruption by ice formation. It appears that biological surfaces exert some influence on the organization of the surrounding water (the so-called hydration layer) over distances of the order 2-3 nm. This produces a local cryoprotection effect, preventing water in channels or cavities smaller than about 5 nm in diameter from crystallizing. Thus, the <u>biological material itself can play the role of cryoprotectant</u>.

The presence of water close by or superimposed upon biological particles is not proof of their being embedded. In particular, when water freezes in large hexagonal crystals, particles have a tendency to be expelled and become concentrated on the surfaces. Because some drying takes place just before freezing, especially when the bare grid method is used, the final concentration of solute in the vitrified state may be significantly higher than in the original suspension and this may affect the properties of the specimen.

Particle dimensions in the vitrified state are close to those in liquid solution. This is somewhat surprising since amorphous ice has a density 5-7% lower than liquid water at room temperature and the supporting aqueous matrix would therefore be expected to expand by about 2% on vitrification. Vitrified water seems to behave like a liquid around the more rigid biological particles. The vitrified state is a good static model of the liquid suspension.

Once the specimen is vitrified, it must be kept below the devitrification temperature, T_d, during all steps including transfer into the microscope. Cryo-transfer devices have been designed to aid in

the transfer of the vitrified specimen into the microscope without ice condensation on the specimen (Fig. II.73). Condensing water forms hexagonal ice whereas vitrified ice turns into cubic ice when warmed above the vitrification temperature. The transformation of vitreous ice into cubic ice is best monitored by electron diffraction since cubic ice gives rise to discrete diffraction maxima whereas vitreous ice only produces broad, diffuse rings in the pattern.

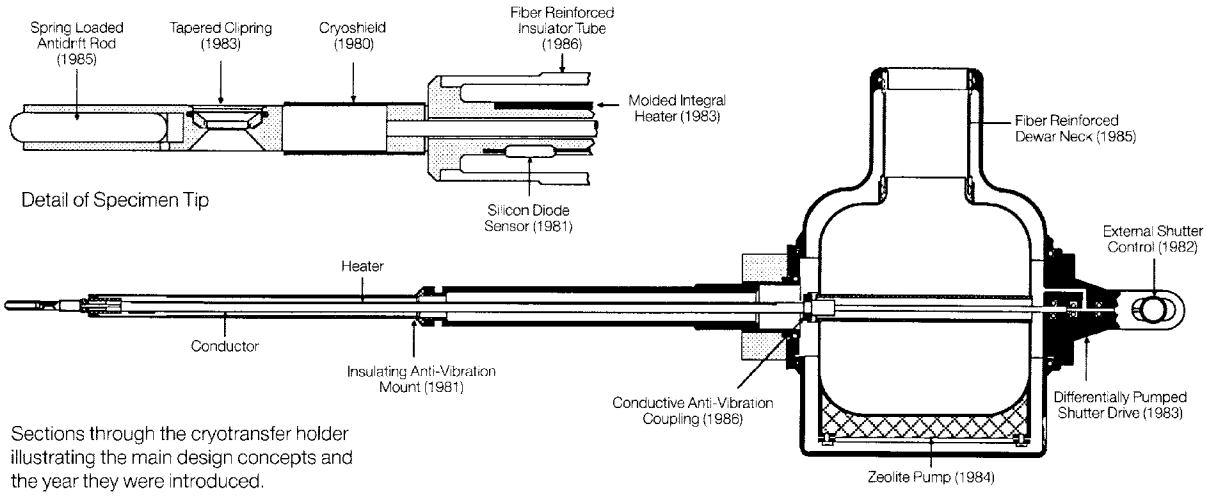


Fig. II.73. Cross-section diagrams through the Gatan Model 626 cryo-transfer holder illustrating the main design concepts and the year they were introduced. (From Gatan, Inc. brochure)

5) Specimen contamination

Because the specimen itself is kept at such a low temperature, it can act as an anticontaminator and residual gases in the microscope will condense on and contaminate the specimen. It is very important therefore, to make certain that the normal anticontaminator in the microscope is used to help trap contamination before it reaches the specimen. In some microscopes, it is necessary to add a second anticontaminator to provide cold surfaces above and below the specimen to assure that the specimen does not become contaminated (see *e.g.* Fig. I.88).

6) Contrast

The inherent contrast of vitrified specimens is about three times lower than that for freeze-dried specimens where there is no surrounding water. Judicious defocusing (under focus) of the objective lens is the best means to control image contrast since images of unstained, vitrified material are dominated by phase contrast. The comparable yet significantly different electron scattering densities of protein, lipid, nucleic acid, and ice dominate contrast in specimens prepared in frozen solution. Since the density of ice is 0.933 g/cm^3, and that of most biological specimens lies in the range 1.3-1.7 g/cm^3, the biological particles appear with positive contrast (Fig. II.74). The density of the aqueous medium can be changed by altering solutes in the medium, which alters the contrast of embedded particles. However, charge interaction at low concentrations of solute may lead to a non-uniform distribution of ions around the particles.

An ideal specimen for both imaging and electron diffraction purposes is one that is embedded in the thinnest possible film of ice that, although surrounding the object, does not actually extend over it with any appreciable thickness. Excess ice over the specimen reduces image contrast. The best images or diffraction patterns appear to be obtained with aqueous films comparable in thickness to the specimen. However, when the thickness of the layer is close to the dimension of the particles, interaction with the surface can become noticeable.

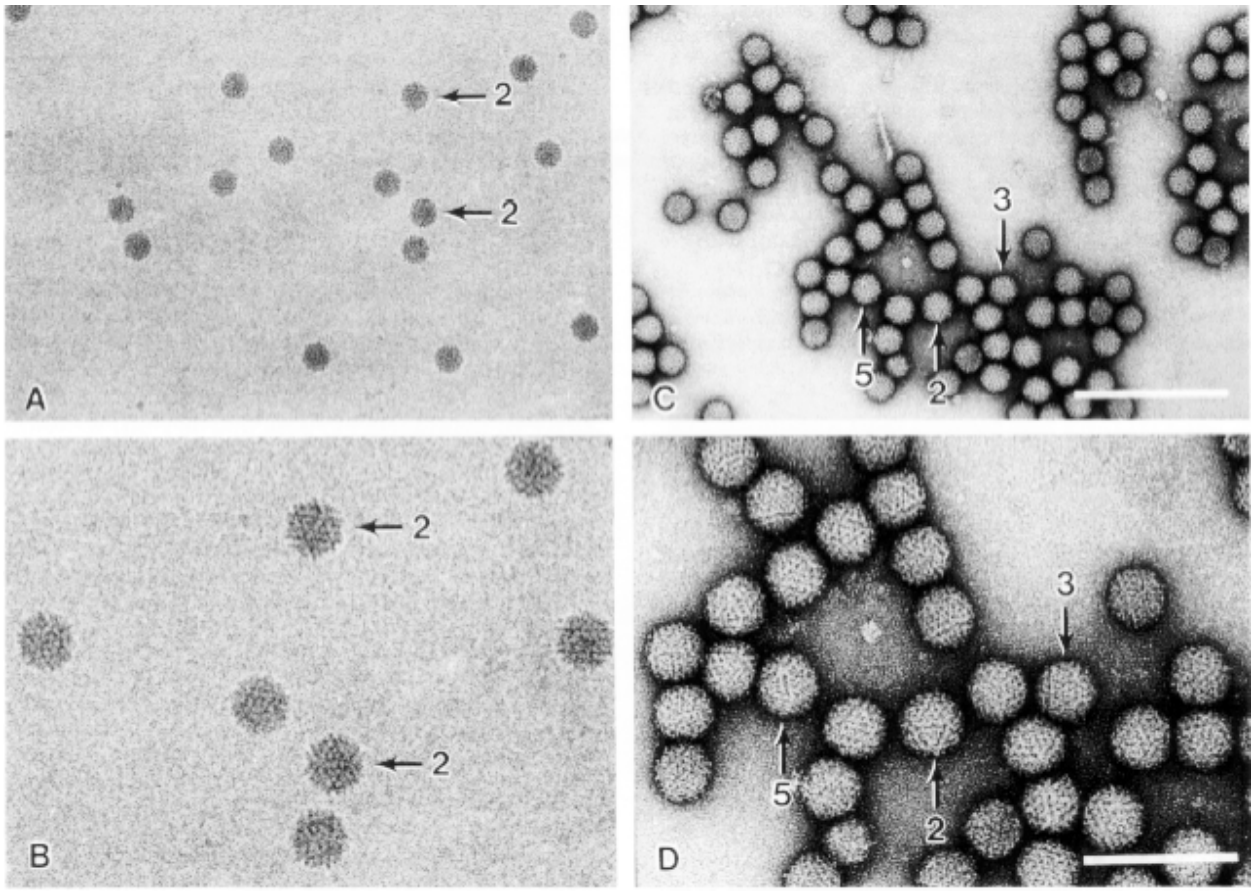


Fig. II.74. Electron micrographs of frozen-hydrated (A and B) and negatively stained (C and D) *Nudaurelia capensis* virus samples. Virions viewed close to icosahedral two-fold (2) and three-fold (3) axes are labeled. The "5" identifies a particle displaying a prominent fivefold vertex in the upper left portion of the particle image. Magnification bars: 200 nm (A,C), 100 nm (B,D). (From Olson *et al.*, 1990, p.114)

7) Radiation sensitivity

The use of vitrified specimens for molecular structure determination is limited primarily by radiation damage. At 100°K (-173°C) vitrified, freeze-dried, or sugar-embedded crystals can withstand on the order of about a ten-fold increase in electron exposure for the same damage when compared with similar sugar-embedded or freeze-dried samples at room temperature. It is thought that the radiation damage protection stems from reducing the temperature-dependent rearrangement or diffusion of fragments resulting from bond fracture (the primary radiation induced event). In the frozen (solid) state, diffusion or rearrangement is reduced and the protein conformation is more likely to be maintained up to higher levels of irradiation.

The three characteristic structural effects of beam damage with respect to vitrified specimens are 1) devitrification, 2) mass loss, and 3) bubbling. In pure water the transition from vitrified to cubic ice can be triggered by the electron beam. The dose needed for this transition increases with decreasing temperature. Mass loss is proportional to the dose but not to the thickness of the irradiated layer. Mass loss is independent of the electron flux and of the size of the irradiated surface. This suggests that the rate-limiting step in mass loss is a surface phenomenon (*i.e.* some kind of evaporation process). A dose of ~60 electrons is needed to remove one water molecule. This is approximately the dose required to produce one inelastic scattering event per molecule. Bubbling occurs after a dose corresponding to about 1,000-10,000 electrons/nm^2 (= 10-100 electrons/$Å^2$) (Fig. II.75). The dose required for bubbling increases with decreasing temperature. It is thought that bubbling is the result of the accumulation of molecular fragments unable to escape from the interior of the specimen.

Radiolysis of ice leads to the formation of very active free radicals such as H• and OH•. Ionization

of ice leads to the formation of H_2O^+ and e_m^- (mobile electrons). Excitation gives a free radical pair H• and OH•. e_m^- may react with water molecules to give H and OH.

Ionic reactions:

$$H_2O^+ + H_2O \quad \text{---> } H_3O^+ + OH\bullet$$

$$H_3O^+ + OH\bullet + e_m^- \text{ ---> } 2H_2O$$

Most of the visible damage in vitrified specimens is observed in regions where organic material and ice are in contact.

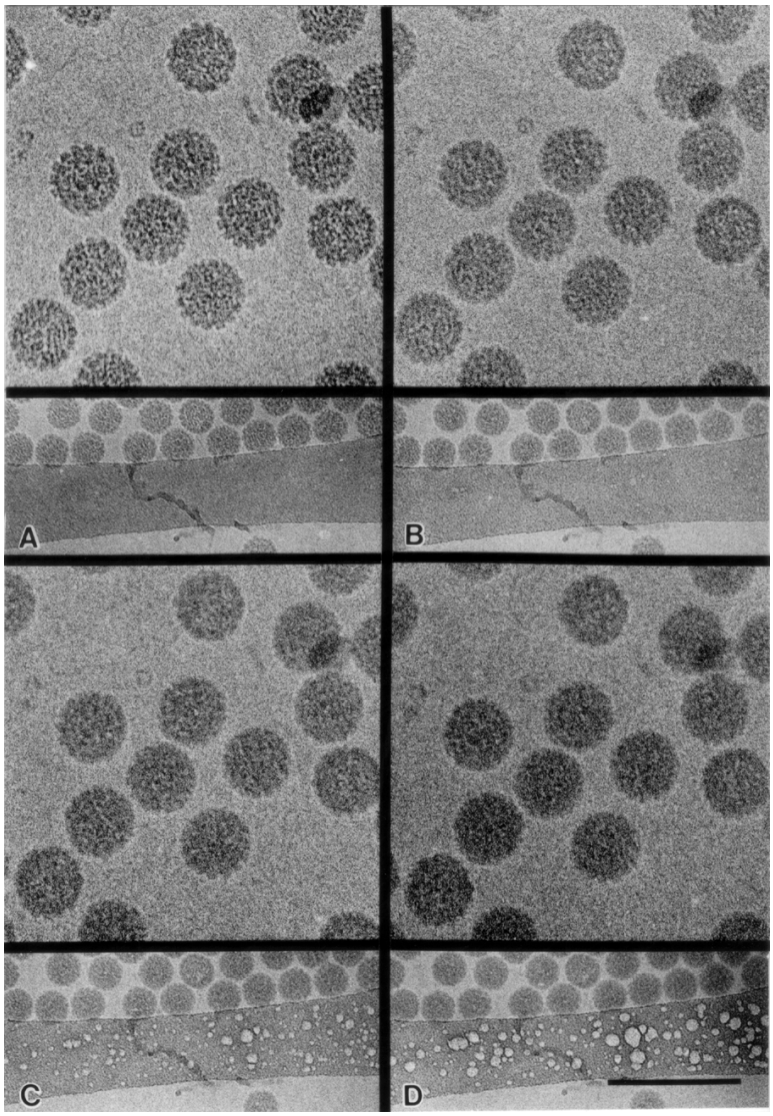


Fig. II.75. Dose series of unstained, vitrified SV40 virus prepared on a holey-carbon film. A. 10 $e^-/Å^2$. B. 20 $e^-/Å^2$. C. 30 $e^-/Å^2$. D. 40 $e^-/Å^2$. The top and bottom images in each panel show high and low magnification views, respectively. Detail features in the virus capsid already begin to disappear after a dose of 20 $e^-/Å^2$. Bubbles form first over the carbon film (C-D). At higher doses (>50 $e^-/Å^2$; not shown) bubbles then begin to appear in the virus particles over the hole. The water over the hole forms bubbles after continued irradiation. Scale bar in (D) represents 200 nm. (Adapted from Baker, Olson, and Fuller, 1999, p.873)

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