HOT ALCOHOLIC PHOSPHOTUNGSTIC ACID AND URANYL ACETATE AS ROUTINE STAINS FOR THICK AND THIN SECTIONS

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One of the potential advantages of high-voltage electron microscopes is that their greater penetrating power allows the use of thick sections (0.2–2 μ) to study three-dimensional cytoarchitecture. So far, this advantage has been limited because conventional methods of staining only allow the penetration of heavy metal solutions into sections to a depth of a few hundred Angstroms. For example, Glauert and Mayo (1970), using a 1 Mev microscope, say ". . . for sections thicker than 0.5 μm stains must be introduced into the section before embedding, since stains will not penetrate the sections, even if the time of staining is prolonged." The problem has been partly solved by staining tissues with hot uranyl acetate before embedment (4) but this treatment only gives marginally adequate contrast at 650 kv (Fig. 11). The object of the work now reported was to find a way by which heavy metal solutions could penetrate tissues already embedded in plastic. A simple method is described for staining material already in resin uniformly to depths of 15 μ to give excellent contrast even for accelerating voltages of 650 kv. The procedure promises to allow the study of biological material with the high-voltage electron microscope in a way not possible hitherto. For thin sections at 100 kv it gives very high contrast uniformly through the section without surface precipitation. The procedure is easy and repeatable. It has also been possible to stain sections embedded in resins which have proved refractory to staining by other methods.

MATERIALS AND METHODS

Insect tissues (5th stage *Calpodes ethlius* larvae) were fixed in 2.5% glutaraldehyde with 0.05 M phosphate buffer at pH 7.2 for 3 hr and postfixed in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate at pH 7.2 for 2 hr. Some material was also fixed according to Karnovsky (3). Tissues were dehydrated in ethanol and embedded in Araldite. Photographs were taken on Kodak contrast plates with an RCA 3F electron microscope operated at 100 kv (35 μ objective aperture) and with a Hitachi high voltage electron microscope operating at 650 kv (20 μ objective aperture). For comparisons of contrast obtained by different staining methods, the exposure and development of plates and prints were carried out under comparable conditions.

The Staining of Sections

Grids with sections are easily manipulated for staining in a plastic ring cut from hard polythene tubing (5). Grids are inserted in nicks cut on the inside of the ring with a thin Gillette razor blade (Fig. 1). This grid holder is dropped into a vial of staining solution warmed to about 60°C in an oven. After staining, the grids are washed for a few seconds by holding the plastic ring with forceps in a gentle stream of rinsing solution from a wash bottle. The solution drains instantly when the ring is touched to filter paper.

Conditions Affecting the Penetration of Stain

Araldite sections approximately 1 μ-thick were mounted on strips of Araldite and immersed in the stain to be tested. They were then re-embedded in Araldite and sectioned normal to the original plane of section. For these experiments 1.5–2% solutions of uranyl acetate (10) and phosphotungstic acid (PTA) (2, 6) were made in water and 95% ethanol. Alcoholic uranyl acetate is light sensitive and throws down a yellowish-brown precipitate after a few days' exposure on the bench. The solution is stable in the dark or after acidification with 0.15 ml glacial acetic acid per 10 ml reagent. Staining was carried out for various times from 2 min to 30 min at about 60°C and at room temperature. Some of the results are shown in Figs. 2, 3, 4, and 5. At room temperature both alcoholic and aqueous solutions caused a precipitate reflecting the distribution of tissue but limited to the outer 500–1000 A (Figs. 2, 3). Hot aqueous stain allowed some penetration together with a surface precipitate. Hot alcoholic stain penetrated through the whole thickness of these sections and selectively stained tissue components with little or no surface

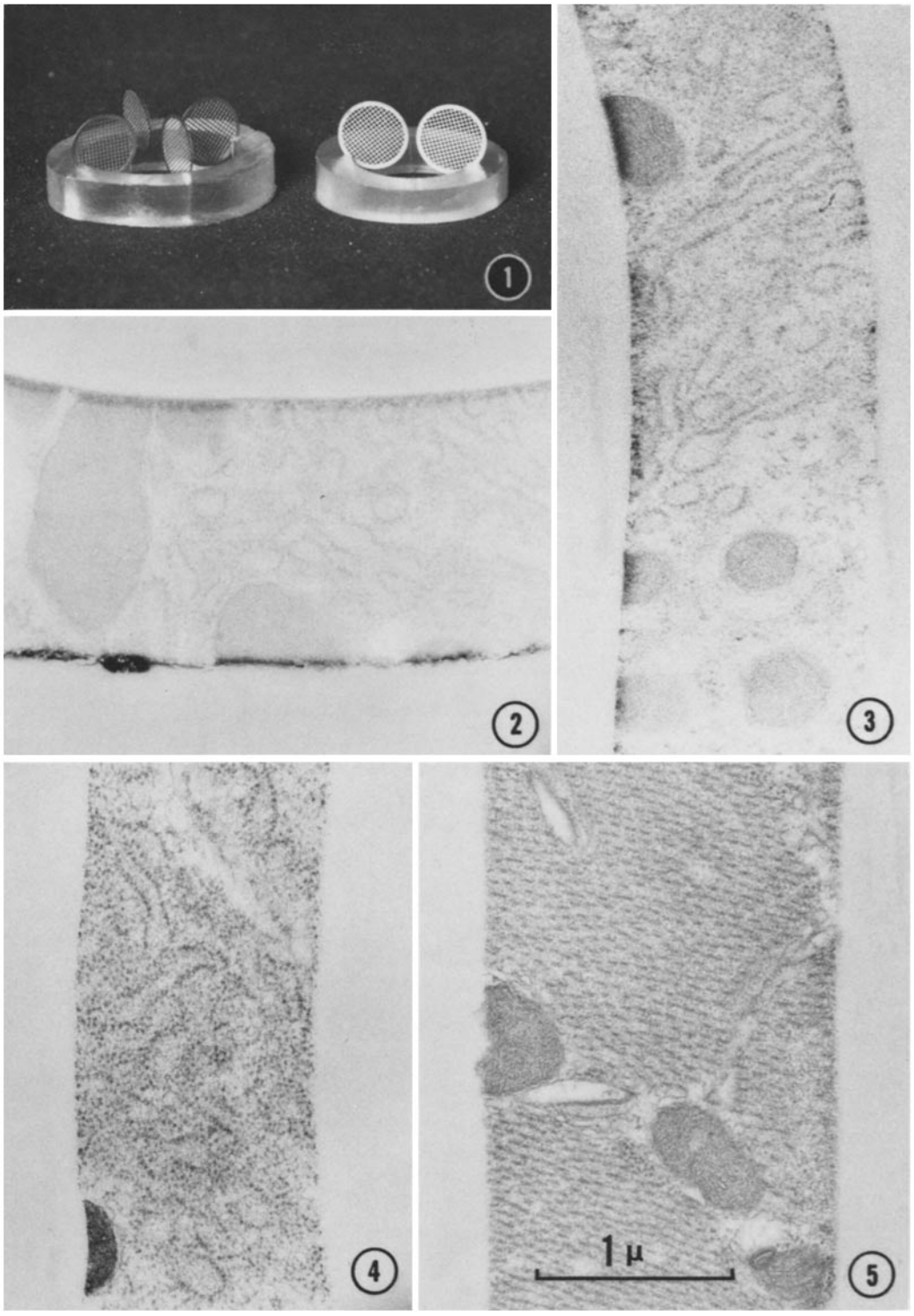
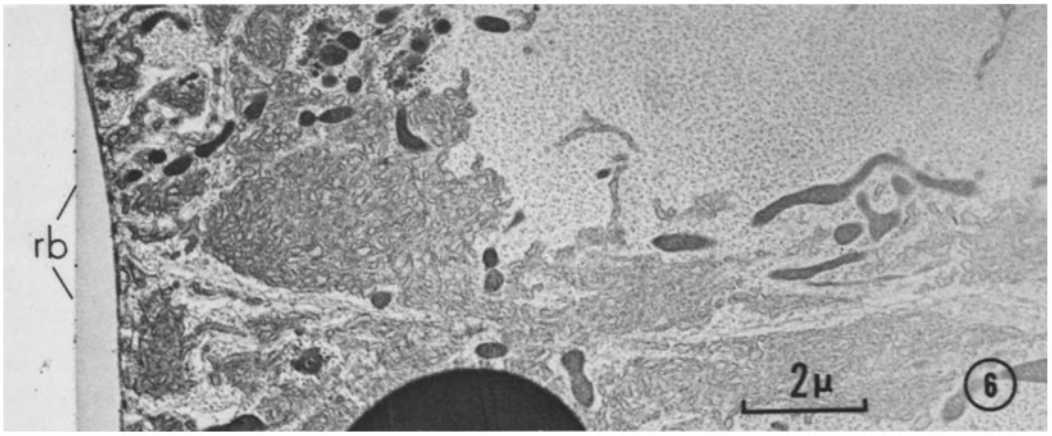


FIGURE 1 The plastic rings used to hold grids for staining (5).

FIGURES 2–5 The penetration of stains at room temperature (Figs. 2 and 3) and at 60°C (Figs. 4 and 5). Thick Araldite sections were stained and then re-embedded and sectioned. Fig. 2: thick section stained for 10 min in 2% phosphotungstic acid in 95% ethanol at about 22°C. The stain is granular and limited to the surface of the section exposed to the stain. Fig. 3: thick section stained for 30 min in 2% aqueous uranyl acetate at about 22°C. The stain does not penetrate much below the surface. Fig. 4: thick section stained for 10 min in 2% phosphotungstic acid in 95% ethanol at 60°C. The stain penetrates uniformly through the section. Fig. 5: thick section stained for 30 min in 1.5–2% uranyl acetate in 95% ethanol at 60°C. The stain is uniformly distributed through the section. Printed on kodabromide F5 paper. × 30,000.



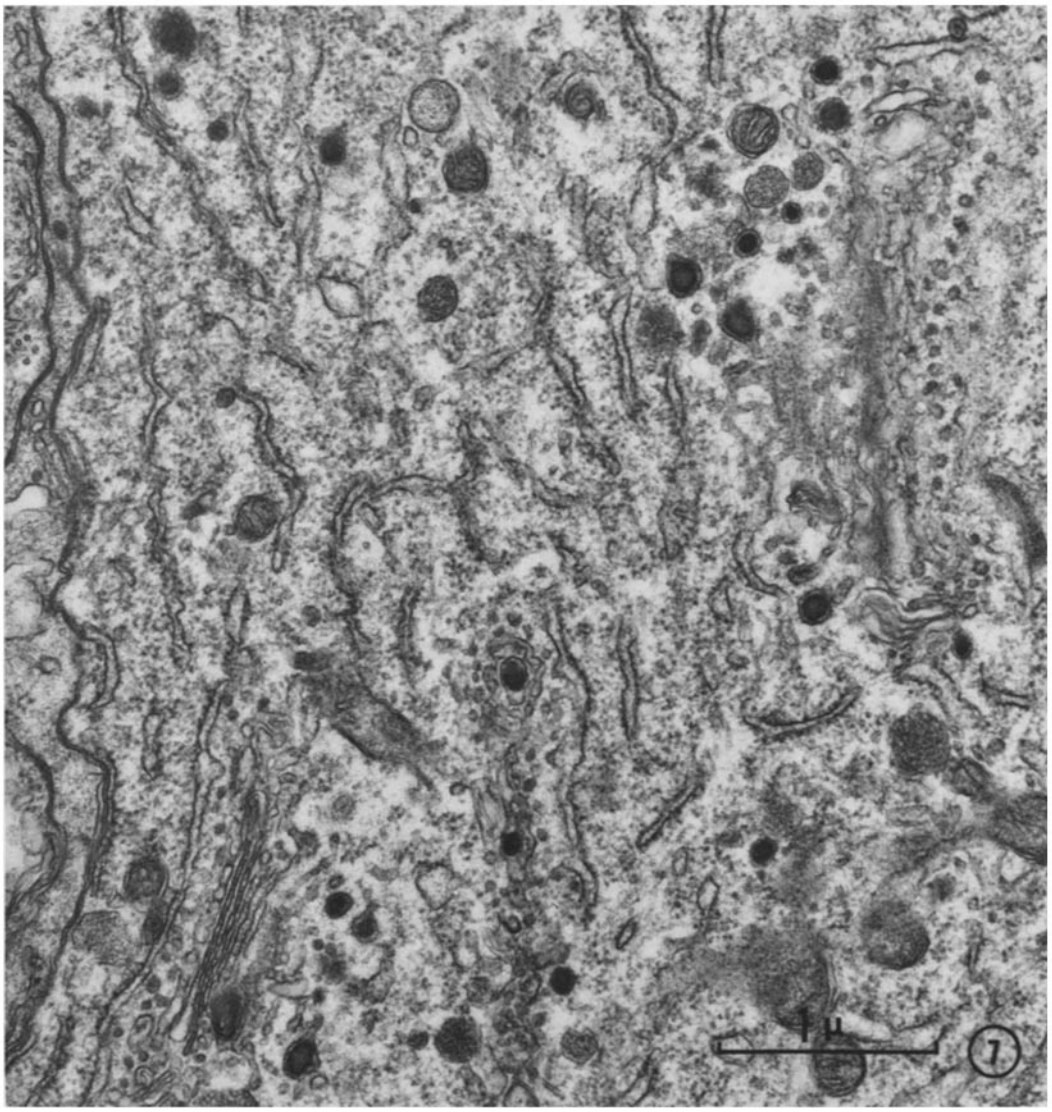


FIGURE 6 The penetration of stain into tissue embedded in Araldite. A block of resin was trimmed to expose tissue (fat body) on one face. The block was then stained in 2% phosphotungstic acid in 95% ethanol for 22 hr at 60°C. Sections were cut normal to the exposed face. PTA penetrates to give good contrast for 10–15 μ. Under similar conditions uranyl acetate penetrates even farther. *rb*, face of resin block exposed to stain. Printed on Kodabromide F3 paper. × 8400.

FIGURE 7 The contrast obtained by staining sections for 10 min with 2% phosphotungstic acid in 95% ethanol at 60°C. Section of a secretory cell in an insect (*Calpodes*) abdominal ganglion. Printed on Kodabromide F3 paper. × 29,000.

precipitate (Figs. 4, 5). In these tests the two heavy metal solutions were similar in their penetration.

The extent of penetration of stain into blocks of embedded tissue was studied further in the following way. Blocks of tissue embedded in Araldite were sectioned to expose tissue on one face and then immersed in the above stains in alcohol at 60°C for about 22 hr. The blocks were then resectioned normal to the exposed surface. Fig. 6 shows that phosphotungstic acid penetrates to give very good contrast for as much as 15 μ and satisfactory contrast to a much greater depth. Uranyl acetate gives excellent contrast for at least 30 μ. The penetration of the stain for many microns into the block makes it possible to stain a block before cutting hundreds of sections of suitable thickness for conventional electron microscopy.

These results suggest that hot alcoholic solutions of stain will improve contrast on the thin sections used for conventional electron microscopy, and that 0.5–2 μ sections can be stained uniformly throughout their thickness for use on the high-voltage electron microscope.

Staining Sections for Conventional Electron Microscopy

500–1000 A-thick sections were stained with uranyl acetate (Fig. 8) or phosphotungstic acid (Fig. 7) in 95% ethanol at 60°C for 10–30 min, or both. A useful treatment also consisted of hot phosphotungstic acid on sections containing tissue which had previously been incubated in hot alcoholic uranyl acetate. For most purposes enough contrast is obtained by either stain on its own. Sections stained throughout their thickness in this way are particularly useful for evenly resolving details of structure which are both at and below the surface of the section. For example, it is very difficult to resolve the banding pattern of collagen fibers from the basement membrane of insects after staining in uranyl acetate (10) and lead citrate (7) at room temperature (Fig. 10) but the pattern is easily resolved after staining sections in hot alcoholic uranyl acetate alone (Fig. 9). The uniformity of staining which this procedure gives should allow reliable quantitative electron microscopy based on summing densities (8, 9).

Staining for High-Voltage Electron Microscopy

Tissues stained with hot uranyl acetate in ethanol (4) have almost enough contrast for high-voltage microscopy (Fig. 11), but this can be enhanced by further staining the sections in hot phosphotungstic acid. Sections were cut with a range of interference colors up to 1.5 μ thick. The thickness was checked by resectioning. The following thicknesses were measured on Araldite sections having particular interference colors.

Blue	3000 A
Yellow	3400 A
1st order red	6500 A
1st order green	7100 A
Colorless, 1 μ setting on LKB ultramicrotone	1.2 μ

The sections were stained in 2% phosphotungstic acid in 95% ethanol at 60°C for 30 min. Figs. 12 and 13 show typical results after this combination of staining tissues in 2% alcoholic uranyl acetate at 60°C overnight followed by staining the sections in hot alcoholic PTA. Photographs were taken with the microscope operated at 650 kv and with a 20 μ objective aperture. Sections of our material in the range 3000–8000 A allowed a good visual appreciation of the relations between elements down to the size of microtubules or mitochondrial cristae. As a rule of thumb, stained sections thicker than 3000 A and with interference colors were useful, but sections without colors were too thick.

The Specificity of Staining

In the past, specific staining for electron microscopy has been hampered because reagents are often destructive to unembedded tissues but fail to penetrate after embedding. Hot alcoholic reagents penetrate without noticeable damage to the tissue and deserve wider use. For example, we have found that hot phosphotungstic acid makes visible a class of fibers in the basement membrane of insects whose presence had not been suspected after other staining procedures.

Staining Sections Refractory to Conventional Methods

Several embedding media from time to time give blocks which stain little or with difficulty. We have found that hot alcoholic uranyl acetate and phosphotungstic acid give good contrast for material embedded in Epon, Maraglas, and Spurr. (Material kindly supplied by Dr. R. D. Goldman.)

CONCLUSION

Sections stained with hot alcoholic solutions are to be preferred over sections stained with aqueous stains at room temperature because they give (*a*) more contrast, i.e. more heavy metal is taken up, (*b*) a more faithful representation of the distribution of cell components since the section is stained uniformly throughout its thickness, (*c*) the possibility of better resolution since the stain within the resin is less granular than that precipitated at

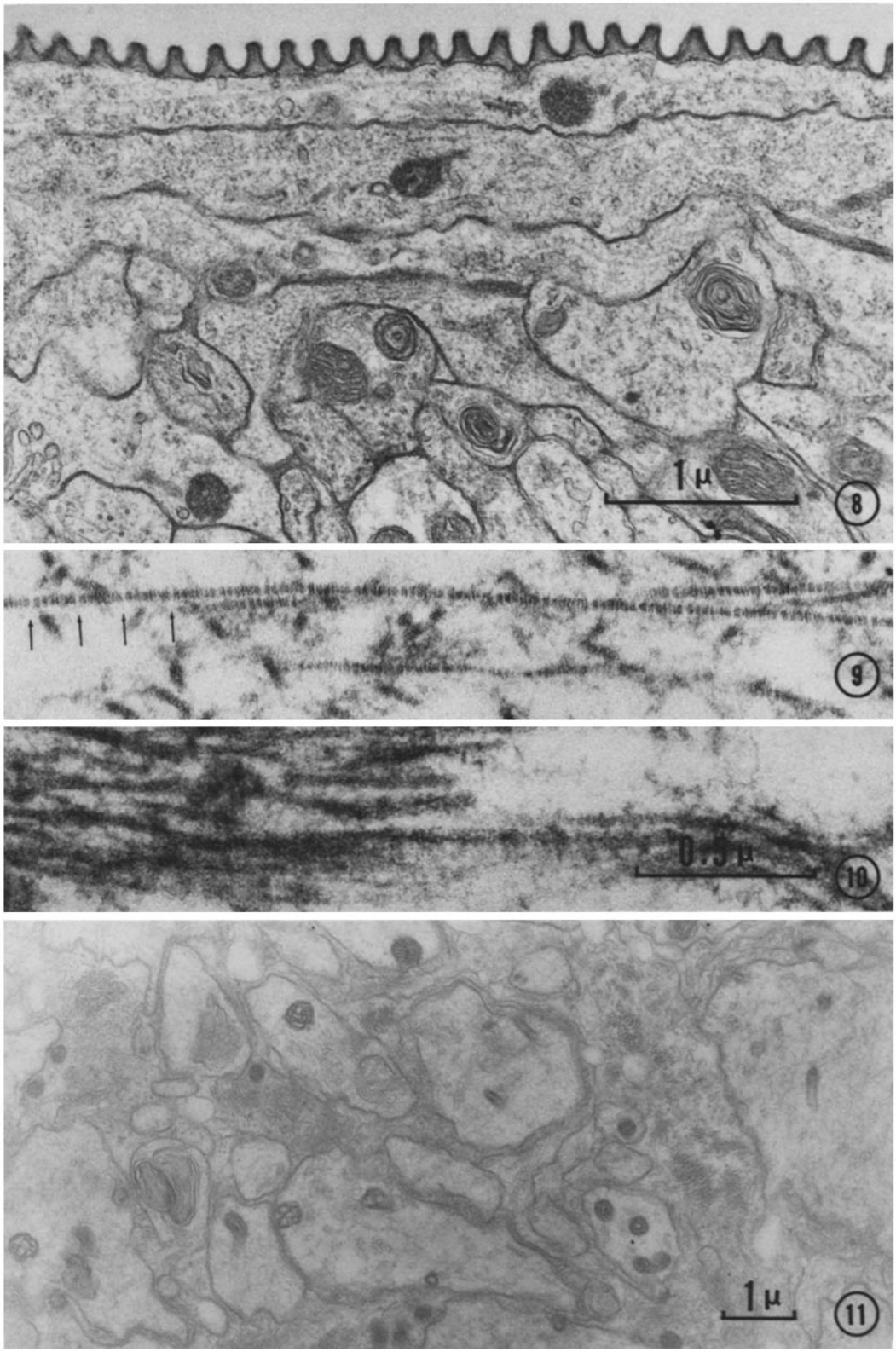
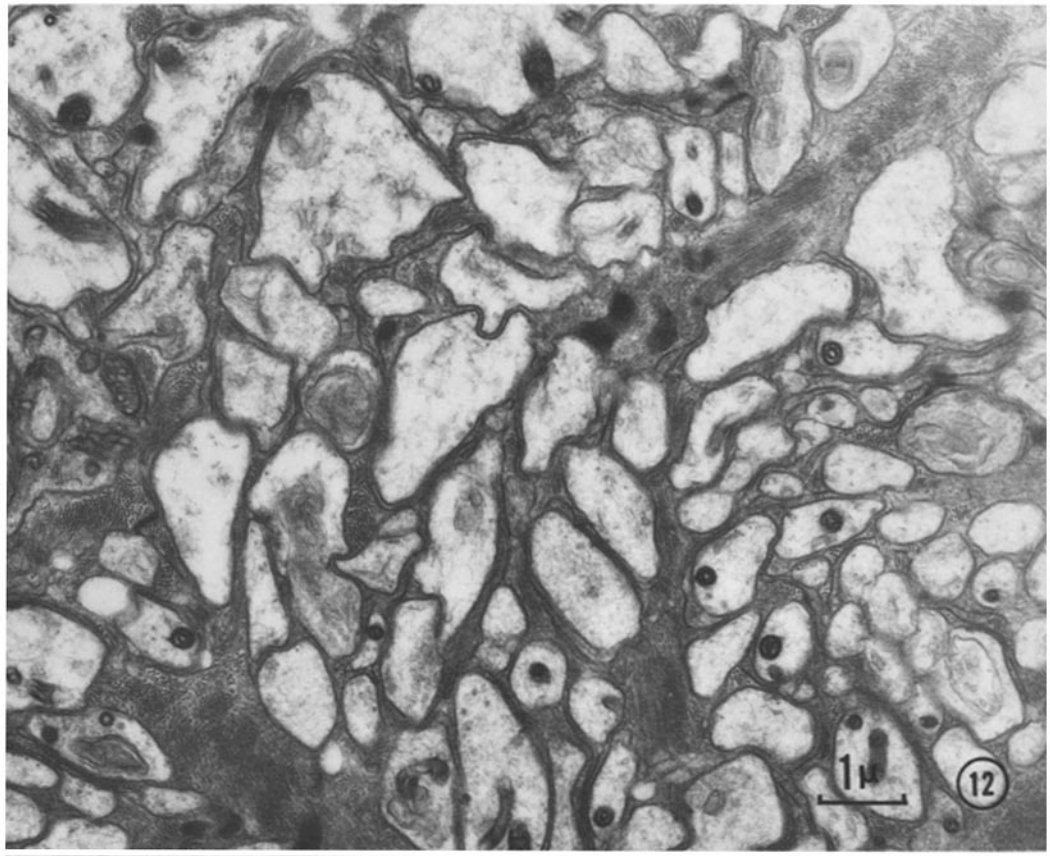
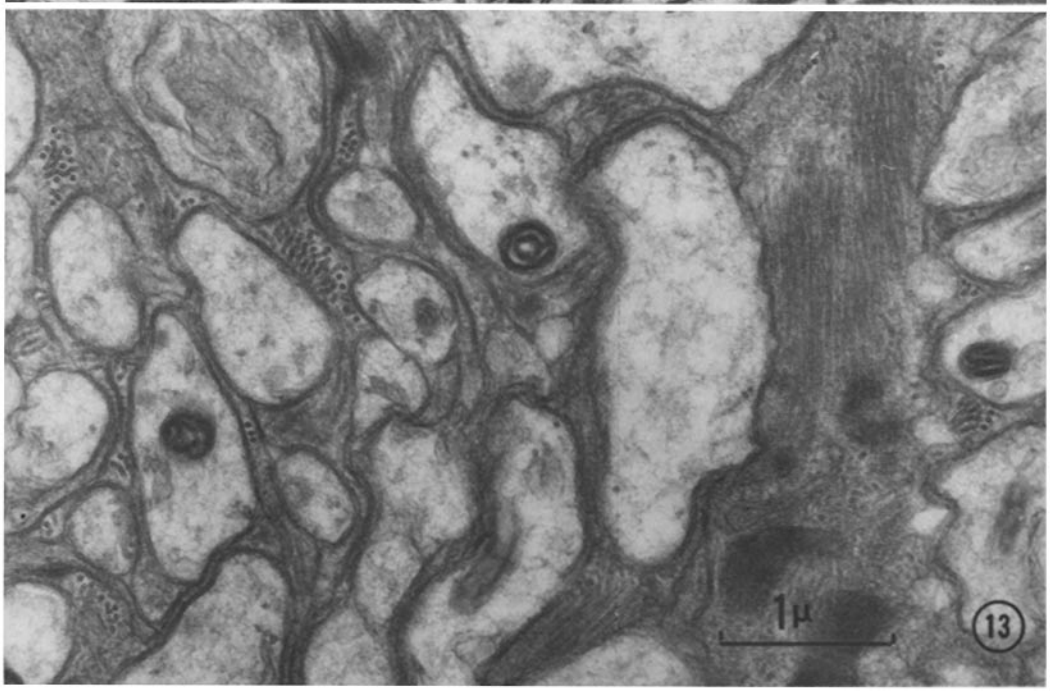

1 μ
8
9
0.5 μ
10
1 μ
11

FIGURE 8 The contrast obtained by staining sections for 30 min with 1.5–2% uranyl acetate in 95% ethanol at 60°C. Section of tracheole cell and nerves. Printed on Kodabromide F3 paper. × 29,000.

FIGURES 9 and 10 Collagen in insect connective tissue. The resolution after staining with hot uranyl acetate compared with conventional staining in uranyl acetate (10) and lead citrate (7) at room temperature. Fig. 9: Section stained for 30 min in 1.5–2% uranyl acetate in 95% ethanol at 60°C. The repeat banding (↑) of collagen within the section is clearly visible. Fig. 10: Section similar to Fig. 9 but stained at room temperature for 10 min in saturated uranyl acetate in 1:1 70% ethanol and methanol, and for 5 min in lead citrate. The pattern of banding is grainy and difficult to resolve. Printed on Kodabromide F5 paper. × 111,000.

FIGURE 11 The contrast obtained at 650 kv after treating tissue for 24 hr in 1.5–2% uranyl acetate in 95% ethanol at 60°C. Sections viewed without further staining. The contrast is scarcely adequate. Section about 3000 A thick. Printed on Kodabromide F5 paper. × 11,000.





FIGURES 12 and 13 The contrast at 650 kv obtained by staining tissue for 24 hr in 1.5–2% aqueous uranyl acetate at 60°C followed by staining the section for 10 min in 2% phosphotungstic acid in 95% ethanol at 60°C. Transverse section of insect nerve cord about 6500 A thick. Tissue components such as microtubules are resolvable with excellent contrast. Even greater contrast can be obtained by further staining the sections in hot alcoholic uranyl acetate. Fig. 12, × 11,000; Fig. 13, × 23,000.

the surface, and (*d*) the possibility of more specific staining.

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