

# POLYESTER-METHACRYLATE EMBEDMENTS FOR ELECTRON MICROSCOPY

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## ABSTRACT

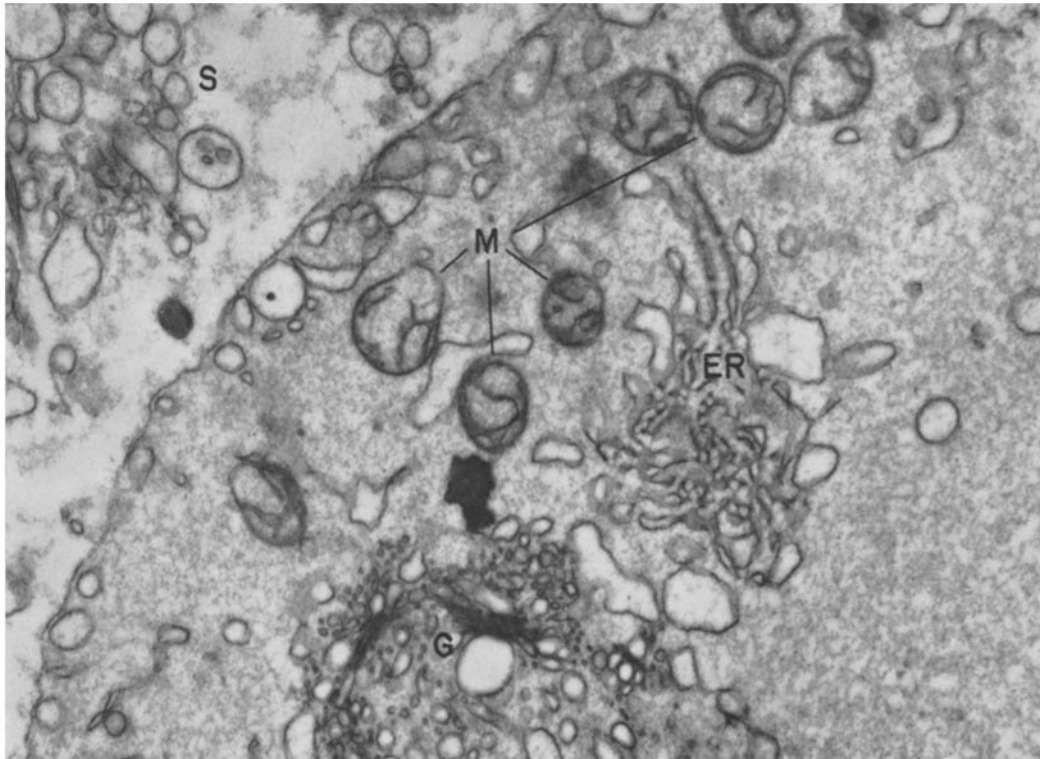
It has been found that tissues fixed for electron microscopy and dehydrated in acetone can be embedded in mixtures of *n*-butyl methacrylate and polyester resin. Activation with 1 per cent tert-butyl hydroperoxide followed by 12 to 48 hours at 60°C produces blocks that section well with glass knives. The ribbons are cleared of methacrylate by heat (200–250°C for 1 hour) and/or immersion in organic solvents (CCl<sub>4</sub>, acetone-ether). After removal of the methacrylate the residual polyester matrix provides thermostable and insoluble support for the tissue. Its insolubility permits staining by immersion of cleared preparations in organic solvents carrying heavy metal compounds in solution. Clearing by heat stabilizes section-grid relationships. The removal of volatile materials by clearing substantially reduces contamination of both specimen and microscope. Tissue fine structure is well preserved in these preparations.

## INTRODUCTION

The plastic embedding media used for electron microscopy now include methacrylates (5, 16), epoxy resins (2, 6, 8, 9, 15), and polyester resins (10, 11, 22). Each kind of embedment has special characteristics that affect its usefulness to the microscopist. Methacrylates cut readily but are thermolabile, and in their use damage may result to tissue fine structure unless special precautions are taken (22, 24). Both epoxy resins (Araldite, Epon) and polyesters (Vestopal-W, Rigolac) are thermostable and can withstand the heat of the electron beam without membrane support. But they infiltrate tissue less readily, are difficult to cut, and sometimes present contrast problems (6, 18). They are likely to be irregular in their responses to routine manipulations, although a recent publication has reported elimination of these difficulties in Araldite and Epon (15). It is evident that both advantages and disadvantages attend the use of any one of the generally known embedding media (18).

Polyester resins possess two characteristics of special interest to the microscopist. First, their monomers have a wide range of miscibility with other substances. Second, their polymers form a reticular matrix that is thermostable and insoluble in organic solvents (1, 3, 7, 22). Their miscibility is shared with the methacrylates (whose polymers are neither thermostable nor insoluble) and their resistant matrix with the epoxy resins (whose monomers have limited miscibilities). Furthermore, the polyester matrix is sufficiently resistant to heat and organic solvents to permit mounted ultrathin sections to be "cleared" of volatile and soluble substances (13). These cleared preparations are then stainable by heavy metal compounds dissolved in organic solvents (13).

It seemed reasonable to expect that the interesting properties of methacrylate and polyester might be retained in mixtures of the two. This paper reports the results of experiments performed to investigate this possibility.



**FIGURE 1**

This field, from mouse testis, illustrates small parts of a spermatid in spermiogenesis, whose dark cytoplasm contrasts with that of an adjacent Sertoli cell (*S*). It was fixed in  $\text{KMnO}_4$  (14), dehydrated in acetone, and embedded in 60 per cent Selectron 5003 and 40 per cent *n*-butyl methacrylate. The sections were mounted on a 200 mesh copper grid covered with a carbon-Formvar membrane. The grid was cleared by immersion in  $\text{CCl}_4$  for  $1\frac{1}{2}$  hours and then in acetone-ether with lead acetate crystals for 7 hours. No heat was used. The section is thick, as indicated by the obliquity of the membranes in the endoplasmic reticulum (*ER*) and the Golgi zone (*G*). The mitochondria (*M*) show no signs of compression.  $\times 20,000$ .

**FIGURE 2**

The acinar cells of monkey pancreas are often characterized by "open" cisternae of endoplasmic reticulum (*ER*). The cisternae in the cell at the lower right are more nearly closed. This tissue was fixed in  $\text{OsO}_4$  (17, 20) and postfixed overnight in neutral buffered 10 per cent formalin. The water used to dilute the formalin contained 0.1 per cent uranyl nitrate. The tissue was dehydrated in acetone and embedded in a mixture containing 10 per cent Selectron 5214, 50 per cent Selectron 5003, and 40 per cent *n*-butyl methacrylate. The sections, mounted on a 500 mesh nickel grid without membrane, were cleared by heating slowly to  $225^\circ\text{C}$ , and held at this temperature for  $1\frac{1}{2}$  hours. The grid was then immersed in  $\text{CCl}_4$  with lead acetate crystals for  $26\frac{1}{2}$  hrs. The long axes of the nucleus (*N*), mitochondria (*M*), and zymogen granules (*Z*) are parallel, indicating some cutting compression.  $\times 20,000$ .



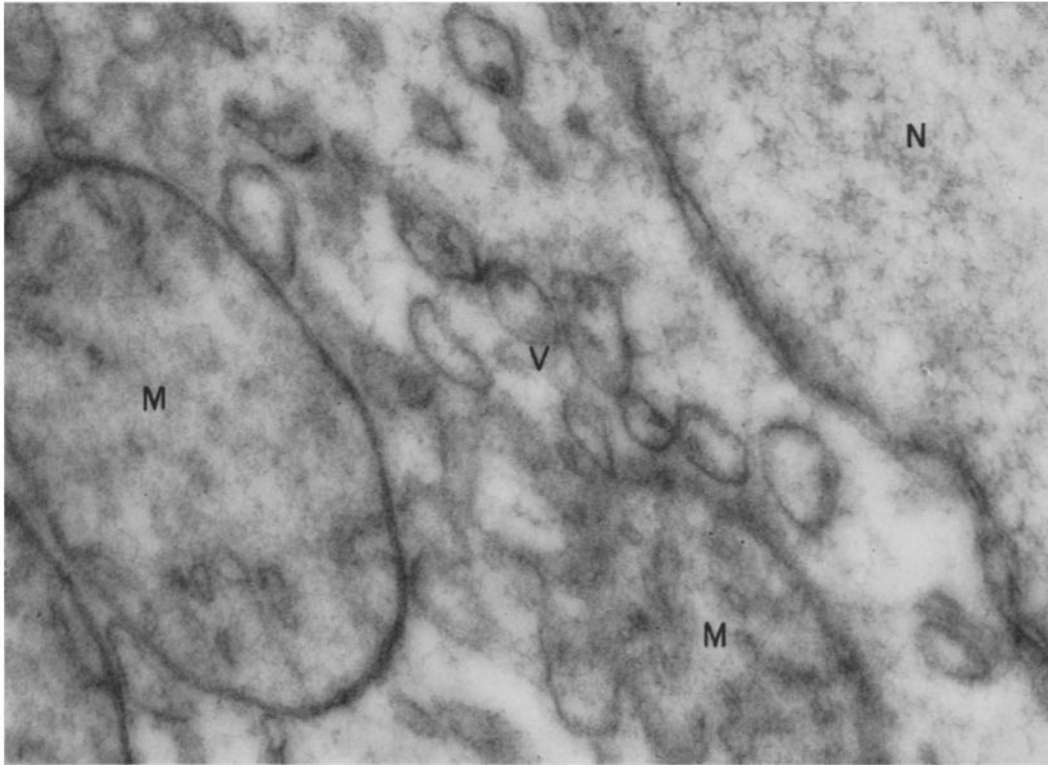


FIGURE 3

This parenchymal cell from mouse liver was routinely fixed in  $\text{KMnO}_4$  (14) but was dehydrated by immersion in glycol methacrylate overnight. It was passed to a mixture containing 60 per cent Selectron 5003, 20 per cent *n*-butyl methacrylate, and 20 per cent glycol methacrylate, in which it was embedded. The 400 mesh grid, on which the sections were mounted without membrane, was first cleared by heating slowly to  $240^\circ\text{C}$ . This temperature was held for 2 hours and the grid was then immersed for 42 hours in acetone-ether. No stain was used. The structural integrity of nucleus (*N*), mitochondria (*M*), and cytoplasmic vacuoles (*V*) is well preserved.  $\times 75,000$ .

#### PROCEDURE AND RESULTS

Two kinds of polyester, Selectron 5003 (hard) and 5214 (soft),<sup>1</sup> were mixed with *n*-butyl methacrylate in various proportions. It was found that as much as 50 per cent *n*-butyl methacrylate could be used without critically affecting the stability of mounted sections during clearing procedures or during actual use in the microscope. The formulas described below were found to be the most useful.

<sup>1</sup>"Selectron" is a proprietary name for 17 types of polyester resin manufactured by the Pittsburgh Plate Glass Co., Pittsburgh, Pennsylvania. Retail sales of 1 gallon cans (minimum) are usually handled by local paint stores operating through Plastic Sales, Paint and Brush Division of this company.

A mixture containing 60 per cent Selectron 5003 and 40 per cent *n*-butyl methacrylate yields blocks which are hard and brittle but which can be sectioned with care. Cutting compression is usually absent (Fig. 1). Sections from these blocks clear well even if thick. Better cutting quality results from the addition of some Selectron 5214 to make a mixture containing 10 per cent 5214, 50 per cent 5003, and 40 per cent *n*-butyl methacrylate. Some cutting compression is noticeable but is slight (Fig. 2). There is no noticeable change in clearing properties or thermal stability. Equal parts of Selectron 5003 and *n*-butyl methacrylate polymerize to blocks whose cutting qualities resemble those of conventional mixtures of methacrylates. There is some thermal instability in the

mounted sections but this can be overcome by using fine mesh grids (500) or membrane support.

Tissue may be fixed by any of the usual methods. No special precautions are required for  $\text{KMnO}_4$  (14) and formalin (12).  $\text{OsO}_4$  fixation (17, 20) sometimes inhibits curing, and the most successful preventive measure is postfixation in 10 per cent neutral buffered formalin for 2 hours or longer. Very small pieces of tissue are less likely to cause trouble.

Specimens are dehydrated in acetone, in concentrations of 30, 60, and 90 per cent, for  $\frac{1}{2}$  hour each. All the water used to dilute the acetone contains 0.1 per cent uranyl nitrate (or acetate) by weight to reduce polymerization damage (23). One hour in pure acetone dried over Drierite and filtered is sufficient to complete the dehydration. It is followed by 1 hour in 50 per cent acetone and 50 per cent monomer mixture. One hour in the pure uncatalyzed monomer mixture is customary but prolonged infiltration does no harm. The actual embedding mixture is catalyzed with 1 per cent tert-butyl hydroperoxide, but the concentration may be varied from 0.5 to 2 per cent. Polymerization takes place in 12 to 48 hours at 60°C. Nitrogen atmosphere (19) is helpful at this stage. The trimmed blocks are cut with a glass knife, the cutting angle of which should be as close as possible to 45°. A very slow cutting motion is used. Usually no membrane support is required if 400 or 500 mesh grids are used.

Dehydration with the water-soluble plastic glycol methacrylate (21) has received limited trial.<sup>2</sup> This compound mixes readily with *n*-butyl methacrylate and the Selectrons. The tissue illustrated in Fig. 3 was passed from glycol methacrylate into a mixture containing 60 per cent Selectron 5003, 20 per cent *n*-butyl methacrylate, and 20 per cent glycol methacrylate, in which it was embedded. The other technical procedures were routine. These blocks section well and respond characteristically to clearing and staining techniques.

Freshly mounted sections of Selectron-methacrylate embeddings can often be used in the micro-

<sup>2</sup>A 200 gm sample of glycol methacrylate was received from CHEMAPOL, Praha, Czechoslovakia, through the courtesy of Mr. J. Šedina. The same compound, manufactured by Rohm and Haas Co., Philadelphia, and marketed by their Special Products Division as 2-hydroxyethyl methacrylate (Hema), has also been used.

scope without further treatment, but their greatest potentialities are developed by subsequent clearing and staining (13). Furnace heat, slowly raised ( $\frac{1}{2}$  to 1 hour) to 200°C and held at 200–250°C for 1 hour, is an effective clearing procedure that stabilizes section-grid relationships, sharpens tissue outlines, and increases contrast. Further increase in contrast can be obtained by immersing the grid in an organic solvent in which a heavy metal compound is dissolved. Lead acetate crystals in  $\text{CCl}_4$  or equal parts of acetone and ether are effective in 2 hours, but staining continued overnight gives positive results without danger of overstaining. Phosphotungstic acid (0.1 per cent in acetone-ether) is effective in 15 to 30 minutes, but care must be taken not to overstain. The grids are examined in the microscope immediately after clearing and staining, since mounted sections tend to deteriorate on standing in the atmosphere.

It is important that the operator avoid hasty judgment of preparations, particularly if they lack adequate contrast and clarity when examined just after sectioning. Judicious use of clearing and staining can transform apparently defective grids into useful and even distinguished preparations. Heat clearing removes most of the volatile material from the preparation (see Discussion below), thereby reducing contamination by the electron beam (4). It is therefore not unwise to clear preparations by heat before the first examination in the microscope. Then the desirability of further clearing or staining can be determined by direct visual observation of the electron image. If the preparations are heat-cleared first, there is little danger that the action of subsequent operations on the same grid will be inhibited by contamination. Successive examinations may be made at discretion. It has been found rewarding over a period of 18 months to treat each grid individually according to its own particular needs.

#### DISCUSSION

The technique described in this paper closely resembles that routinely used for methacrylate embedding, and differs mainly in the addition of the polyester. However, the significant characteristics of the final embeddings are traceable to *both* of the ingredients. The methacrylate softens the blocks so that their cutting qualities resemble those of 6 to 1 mixtures of *n*-butyl and methyl methacrylate. The methacrylate also plays a significant role in later stages of the technique, since improved

specimen quality for electron microscopy results from removal of its volatile fraction during clearing by heat. For example, when gross blocks of polyester-methacrylate are heat-cleared, it becomes evident that each ingredient of the final block retains the characteristics of its own polymer. Clearing by heat causes discs of Selectron-methacrylate ( $2 \times 7$  mm) to lose considerable weight. The percentile weight loss is determined by the relative amounts of methacrylate (90 per cent volatile) and polyester (*ca.* 10 per cent volatile). These discs do not change in size or shape. The heat drives off volatile substances, chiefly methacrylate, but leaves most of the polyester in place (4). Essentially the same changes must occur to produce the observed improvement in contrast that takes place when mounted sections are cleared by heat. The clearing process can be further extended by immersing heat-cleared grids in organic solvents. Any residual materials soluble in the organic compounds used are thereby removed. The tissue can also be stained at this stage if heavy metal compounds are dissolved in the fluid. During all these operations the polyester matrix remains undisturbed because of its resistance to heat and organic

solvents. It therefore continues to provide support for the tissues.

The characteristics of the polyester-methacrylate embedding technique may be summarized as follows. Tissue sections remain unharmed after removal of as much as 50 per cent of the plastic. They are then readily susceptible to chemical influences upon immersion in a suitable fluid. Unwanted solvent action may be avoided (during early preparation) by dehydration in water-soluble methacrylate. Considered together, these characteristics present a wide spectrum of possible approaches to the analysis of ultrastructure.

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