New Method of Embedding with GMA, Quetol 523 and Methyl Methacrylate for Light and Electron Microscopic Observation of Semi-thin Sections

By

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Summary: A new method of embedding with GMA, Quetol 523 and methyl methacrylate for light and electron microscopic observation of semi-thin sections was devised for easy embedding, infiltration, sectioning and staining. The method employed purified GMA (glycol methacrylate), Quetol 523 and methyl methacrylate with QCU-1 (2, 2'-azobis isobutyronitrile paste) as a catalyst. A mixture of these materials could be polymerized at 60°C for about 12 hr to produce a block with excellent cutting properties.

Tissues were fixed in 2.5% glutaraldehyde with buffered phosphate at pH 7.4 for 3 hr or 2% glutaraldehyde-4% paraformaldehyde with buffered phosphate at pH 7.4 for 3 hr. Sections 0.2-0.5 μm thick were cut with glass knives using troughs on a conventional ultramicrotome. They could be attached to slide glasses or grids by water flotation, without adhesive. They should be dried at room temperature. Staining with aqueous solutions of basic and acid dyes, without removal of the embedding matrix, was sharp and brilliant as usual. These stained sections were observed under a light microscope. Identical sites on such sections, 0.2-0.3 μm thick, could be examined with an accelerating potential of 100 kV at low magnification (250-1,500 times) using an LEM-2000 combined light and electron microscope. Thus, photomicrographs and electron micrographs of identical sites on tissue samples could be compared exactly.

The resolution of the electron microscope was so high that the cytoplasmic components were readily identified in the cytoplasm. Osmium tetroxide vapor staining gave better contrast in the images of the specimens.

Introduction

Several embedding methods employing GMA and Quetol 523 have been developed recently for light and electron microscopic observation of semi-thin sections. These methods used only water-miscible methacrylates, GMA and Quetol 523, with QCU-1 as a catalyst. It was difficult to cut 0.3-0.5 μm sections with glass knives using a trough on a conventional ultramicrotome, since the
polymerized blocks were hydrophilic. It was, however, difficult to treat them.

In order to cut semi-thin, 0.2-0.5 μm sections easily with glass knives using troughs on a conventional ultramicrotome as usual, a new method was devised for easy embedding, infiltration, sectioning and staining. This method employed purified GMA, Quetol 523, Quetol 523M [GMA-Quetol 523 (8 : 2) mixture], methyl methacrylate (MMA) with QCU-1 (2, 2’-azobis isobutyronitrile paste) as a catalyst, a mixture of which could be hardened to give excellent cutting properties. The hardness could readily be adjusted by altering the ratio of components to suit the specimens embedded in the mixture. Sections 0.2-0.5 μm thick could then easily be cut with glass knives using troughs on a conventional ultramicrotome as usual. Staining with aqueous solutions of basic and acid dyes, without removal of the embedding matrix, was sharp and brilliant as usual. These stained sections were observed under a light microscope. Identical sites on such sections, 0.2-0.3 μm thick, could be examined with an accelerating potential of 100 kV at low magnification (250-1500 times) using an LEM-2000 combined light and electron microscope. The contrast in the images of the specimens could be improved by exposure to osmium tetroxide vapor.4)11)

### Materials and Methods

1. Specimens

   Fresh tissues (liver, pancreas, small intestine, kidney, salivary gland and testis) excised from adult male I.C.R. mice were used. The tissue sample sizes were approximately 3×3×1 mm.

2. Fixation

   The tissue pieces were fixed in 2.5% glutaraldehyde with buffered phosphate at pH 7.4 or 2% glutaraldehyde-4% para-formaldehyde with buffered phosphate at pH 7.4 for 3 hr at 0°-4°C, and washed in the buffer for 1 hr.

3. Dehydration

   After washing, they were dehydrated in the ordinary way in 50%, 70%, 80%, 90%, 95%, and two changes of 100% alcohol for 30 min each.

4. Embedding

   As mentioned above, the embedding method used purified GMA*, Quetol 523*, Quetol 523M [GMA-Quetol 523 (8 : 2 mixture)]* and MMA with QCU-1* as a catalyst.

   The following mixture was recommended for general embedding:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMA</td>
<td>68 ml</td>
</tr>
<tr>
<td>Quetol 523</td>
<td>2 ml</td>
</tr>
<tr>
<td>MMA</td>
<td>30 ml</td>
</tr>
<tr>
<td>QCU-1</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

   This mixture set in about 12 hr at 60°C yielding clear, colorless blocks. It was suitable for cutting 0.2-0.5 μm sections.

   For preparation of the embedding medium using Quetol 523M, the following formula is recommended.

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMA</td>
<td>60 ml</td>
</tr>
<tr>
<td>Quetol 523M</td>
<td>10 ml</td>
</tr>
<tr>
<td>MMA</td>
<td>30 ml</td>
</tr>
<tr>
<td>QCU-1</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

   After dehydration, the following infiltration procedure was appropriate. All steps were carried out on a shaker at room temperature.

   1) 100% alcohol/GMA-Quetol 523-MMA mixture (1 : 1) 1-2 hr
   2) GMA-Quetol 523-MMA mixture 2-4 hr
   3) GMA-Quetol 523-MMA mixture 2-4 hr
   4) Embed in gelatin capsules

* Sold by Nissin EM Co. Ltd., Esupowaru Ichigaya Bldg., 40-10 Tomihisa-cho, Shinjuku-ku, Tokyo 160, Japan; and Ted Pella Inc., P.O. Box 510, Tustin, Calif. 92680, U.S.A.
Gelatin capsules were filled to the brim with GMA-Quetol 523-MMA mixture, and the specimens were introduced. Polymerization was achieved in about 12 hr at 60°C.

5. Trimming and Sectioning

After removal of the gelatin capsule, the block was placed in a holder and observed under a dissecting microscope to aid precise trimming. The block face was carefully trimmed to a size of ca. 2 x 2 mm with a clean, sharp razor blade. The trimmed block was cut with a glass knife of approximately 10 mm in width using a trough on an ultramicrotome (Porter-Blum MT-1 or MT-2B) or JB-4 Porter-Blum microtome. The sections were generally cut at 0.5 μm in thickness for light microscopy and 0.2-0.3 μm in thickness for electron microscopy.

The meniscus must be kept minimal. Blocks of this resin mixture could be sectioned easily with a glass knife without wetting of the block face. After cutting, each section was spread flat on the water surface in the trough. It was placed on a clean distilled water surface using a platinum loop at room temperature. For electron microscopy, a 50-100 mesh rhodium coated grid was pressed gently over the floating sections in the trough. The excess water on the grid was blown off with a quick blast of clean dry gas from a freon gas spray. The sections were mounted with a cover glass and mounted with Diatex or Eukitt.

For staining the sections on grids, a method employing a short piece of polyethylene tubing as a grid-holder was used to facilitate handling of the grids. The grids were firmly inserted into small slits in the polyethylene tubing and pieces of the tubing were attached to sharp glass rods of a cap. The sections were stained with aqueous solutions of basic and acid dyes without removal of the embedding matrix.

The grids with sections were placed into a staining solution for a given length of time. After staining, they were washed with distilled water and treated with another solution in the case of double staining without drying. After washing with distilled water, the pieces of polyethylene tubing were removed from the glass rods, rinsed carefully with a stream of distilled water directed along the axis of the tubing, and then dried under a gentle jet of freon gas applied in the same direction.

For osmium tetroxide vapor staining, the pieces of polyethylene tubing with the grids were attached to sharp glass rods of a cap. Next, a crystal of osmium tetroxide was placed at the bottom of a vial. The cap with the pieces of polyethylene tubing was placed on the vial and the sections on the grids were exposed to the osmium tetroxide vapor for 3-6 hr.

6. Staining

The sections were stained with aqueous solutions of basic and acid dyes for light microscopy without removal of the embedding matrix. The staining time required was longer than that for paraffin sections.

A drop of staining solution was placed on the section for a given length of time. The excess stain solution was then rinsed away with a jet of tap water, and a drop of the next staining solution was added in the same manner. After a final rinsing in distilled water, the excess water on the slide was blown off with a quick blast of clean dry gas from a freon gas spray. The sections were covered with a cover glass and mounted with Diatex or Eukitt.

For staining the sections on grids, a method employing a short piece of polyethylene tubing as a grid-holder was used to facilitate handling of the grids. The grids were firmly inserted into small slits in the polyethylene tubing and pieces of the tubing were attached to sharp glass rods of a cap. The sections were stained with aqueous solutions of basic and acid dyes without removal of the embedding matrix.

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7. Microscopic Examinations

The stained sections were observed under a light microscope. Identical sites
on such sections, 0.2-0.3 μm thick, were examined with an accelerating potential of 100 kV at low magnification using an LEM-2000 combined light and electron microscope. Electron micrographs were prepared at low magnification (250-1,500 times) and enlarged photographically to a magnification of ca. 3,000 times.

**Results and Discussion**

Several embedding methods employing GMA and Quetol 523 for light and electron microscopic observation of semi-thin sections have used only water-miscible methacrylates, GMA and Quetol 523, with QCU-1 as a catalyst. Semi-thin sections were cut with dry glass knives on an ultramicrotome, since the polymerized blocks were hydrophilic. It was, however, found difficult to treat 0.3-10.5 μm sections by dry cutting.

The present method used purified GMA, Quetol 523 and MMA with QCU-1 as a catalyst. Quetol 523 is a light-colored, flexible, water-miscible methacrylate. It has a low viscosity, and reacted with the GMA and MMA to become an integral part of the polymerized system. The QCU-1 was readily miscible with the methacrylates.

The GMA-Quetol 523-MMA mixture set in about 12 hr at 60°C. It had a low viscosity, and was therefore easy to handle. It penetrated readily and completely into the specimen, producing a homogeneous block from which 0.2-0.5 μm sections could be easily cut. The final hardness of the block could be readily adjusted by altering the ratio of GMA and Quetol 523 to suit the specimen. A mixture of GMA, Quetol 523 and MMA in the ratio of 68 : 2 : 30 was suitable for sectioning at 20°C. The polymerized blocks were reduced in affinity for water, so that it was easy to cut 0.2-0.5 μm sections by wet cutting.

A gelatin capsule with a flat bottom was appropriate for flat embedding of a specimen. A polyethylene capsule was not employed for embedding, since polymerization of the mixture did not proceed to completion in the presence of air.

The tissues were fixed in formaldehyde and glutaraldehyde fixatives, Zenker's fixative, Bouin's fixative, Carnoy's fixative, etc. However, various fixatives containing osmium tetroxide could not be used, since polymerization was retarded or even inhibited in the presence of materials preserved in such fixatives.

Sections 0.2-0.5 μm thick could be easily cut with glass knives using troughs on a conventional ultramicrotome. The sections on the slide or the grid were allowed to dry at room temperature. They adhered to the slide or the grid without adhesive during staining and washing.

For staining sections on grids, a method employing a short piece of polyethylene tubing as a grid-holder was suitable to facilitate handling of the grids. Staining with aqueous solutions of basic and acid dyes, without removal of the embedding matrix, was sharp and brilliant as usual, as illustrated in Figure 1. Figures 9A and 10A depict mouse small intestine and mouse seminiferous tubules. These 0.25-0.3 μm sections were treated with osmium tetroxide vapor after hematoxylin and eosin. Figures 9B and 10B show electron micrographs of identical sites to the sections shown in Figures 9A and 10A, respectively. Figures 9C and 10C are enlargements from Figures 9B and 10B, respectively. Photomicrographs and electron micrographs of identical sites on the tissues could thus be compared exactly.

In Figure 9B, the specific tissue contrast was based on the selected color of the dye at the light microscopic level. In Figure 10B, the specific contrast at the intercellular boundaries distinguished each
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cell and the cytoplasmic components were clearly recognizable. The resolution of the electron microscope was so high that the cytoplasmic components, particularly mitochondria, Golgi apparatus and endoplasmic reticulum, could be readily identified. The cellular structure of the striated border of intestinal epithelia cells, the brush border or proximal tubules of the kidney and desmosomes of the seminiferous epithelium of the testis, were also clearly demonstrated under the electron microscope. Images from osmium tetroxide vapor staining showed a distinctly improved contrast.

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References

Examination of Figures

Plate I

Fig. 1. Mouse small intestine fixed in 4% formaldehyde-2% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with toluidine blue.

Fig. 2. Mouse kidney fixed in 4% formaldehyde-2% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained by the Giemsa method.

Fig. 3. Mouse kidney fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with acid fuchsin.

Fig. 4. Mouse kidney fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with Mallory-Azan.
Plate II

Fig. 5. Mouse small intestine fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with hematoxylin and eosin.

Fig. 6. Mouse seminiferous tubule fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with hematoxylin and eosin.

Fig. 7. Mouse small intestine fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with PAS and counter-stained with hematoxylin.

Fig. 8. Mouse seminiferous tubule fixed in 2.5% glutaraldehyde and embedded in a GMA-Quetol 523-MMA mixture. 0.5 μm section stained with PAS.
Plate III

Fig. 9A. Mouse small intestine fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4) and embedded in a GMA-Quetol 523-MMA mixture. 0.3 μm section stained with hematoxylin and eosin. ×300

Fig. 10A. Mouse seminiferous tubules fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.4) and embedded in a GMA-Quetol 523-MMA mixture. 0.25 μm section stained with hematoxylin and eosin. ×300

Figs. 9B and 10B. Electron micrographs of identical sites to the sections in Figs. 9A and 10A, respectively. These sections treated with osmium tetroxide vapor after hematoxylin and eosin staining. ×300 and ×350 respectively
Plate IV

Figs. 9C and 10C. Electron micrographs, enlarged from Figs. 9B and 10B, respectively \( \times 1,900 \) and \( \times 3,000 \) respectively.