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A Low-Viscosity Epoxy Resin Embedding Medium for Electron Microscopy

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A low-viscosity embedding medium based on ERL-4206 is recommended for use in electron microscopy. The composition is: ERL-4206 (vinyl cyclohexene dioxide) 10 g, D.E.R. 736 (diglycidyl ether of polypropylene glycol) 6 g, NSA (nonenyl succinic anhydride) 26 g, and S-1 (dimethylaminoethanol or DMAE) 0.4 g. The medium is easily and rapidly prepared by dispensing the components, in turn by weight, into a single flask. The relatively low viscosity of the medium (60 cP) permits rapid mixing by shaking and swirling. The medium is infiltrated into specimens after the use of any one of several dehydrating fluids, such as ethanol, acetone, dioxan, hexylene glycol, isopropyl alcohol, propylene oxide, and tert.-butyl alcohol. It is compatible with each of these in all proportions. After infiltration the castings are polymerized at 70°C in 8 hours. Longer curing does not adversely affect the physical properties of the castings. Curing time can be reduced by increasing the temperature or the accelerator, S-1, or both; and the hardness of the castings is controlled by changes in the D.E.R. 736 flexibilizer. The medium has a long pot life of several days and infiltrates readily because of its low viscosity. The castings have good trimming and sectioning qualities. The embedding matrix of the sections is very resistant to oxidation by $KMnO_4$ and $Ba(MnO_4)_2$, compared with resins containing NADIC methyl anhydride. Sections are tough under the electron beam and may be used without a supporting membrane on the grids. The background plastic in the sections shows no perceptible substructure at magnifications commonly used for biological materials. The medium has been used successfully with a wide range of specimens, including endosperms with a high lipid content, tissues with hard, lignified cell walls, and highly vacuolated parenchymatous tissues of ripe fruits.

Among the newer epoxy resins, ERL-4206 provides exceptional qualities for use in an embedding medium for electron microscopy. In particular, its very low viscosity of 7.8 centipoise (cP) conveys a marked reduction in viscosity to epoxy resin mixtures. In selecting other components of the medium, primary consideration was

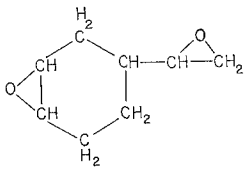
also given to their viscosity. As a consequence the final mixture, with accelerator, is much less viscous than formulations based on Araldite (*5*, *6*), Epon 812 (*6*, *12*), or Maraglas (*3*, *7*, *16*).

MATERIALS

Some of the components of the medium apparently have not been reported in previous formulations. Accordingly technical information on each and the reasons for their selection are presented.

Epoxy resin

ERL-4206, vinyl cyclohexene dioxide, is a cycloaliphatic diepoxide (*18*) with the following structure:



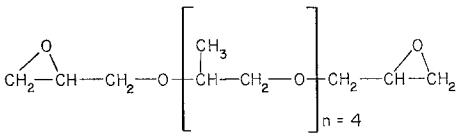
It is a clear, mobile liquid with a molecular weight of 140.18, an epoxide equivalent of 74–78 (grams resin containing one gram equivalent of epoxide), and a specific gravity of 1.10 at 20°C. In some uses it is regarded as a reactive diluent for viscosity control. The low molecular weight and low viscosity probably enhance its penetration into tissues. Its viscosity of 7.8 cP is markedly lower than that of Epon 812 (150–210 cP), Maraglas 655 (500 cP), or Araldite 502 (3000 cP). The complete mixture, with a viscosity of 60 cP at 25°C, is considerably less viscous than Epon 812 alone. Twenty-four hours after preparation, the viscosity of the mixture is 140 cP at 25°C. As the primary epoxy resin in the embedding medium, ERL-4206 lowers the viscosity without sacrificing heat resistance or causing softening of the castings. In fact, in relation to the quantity used in the embedding formulation, it conveys unusual heat resistance to the sections under the electron beam. According to Trigaux (*18*), its compact diepoxide structure yields linear polymers that are highly cross-linked and, as a consequence, have high deflection temperatures (DT). Some of its properties are attributed to a slight difference in chemical reactivity of its two epoxides. In this regard it is more reactive with acid anhydride hardeners than are epoxy resins based on bisphenol A and epichlorohydrin. This may permit more rapid and complete cures. Use cork stoppers for bottles from which ERL-4206 and the D.E.R. 736 are dispensed, as the plastic liners in some screw caps are soluble in these resins. ERL-4206 is moderately toxic, causing a dermatitis. It is reported to have an LD_{50} of 2.83 g/kg on ingestion in rats (*9*). Areas of the skin contacted by the resin or its mixture with other materials should be washed with soap and water—*not* with a solvent. The resin should be used only in areas with adequate ventilation and repeated contact with the liquid should be avoided.

Flexibilizer

ERL-4206 used alone with a curing agent in some systems may result in extremely hard castings and consequently a flexibilizer is required. An epoxy resin, D.E.R. 736 (diglycidyl

ether of polypropylene glycol), was selected as the flexibilizer because of its moderately low viscosity of 30–60 cP at 25°C. It is used in some applications as a reactive diluent.

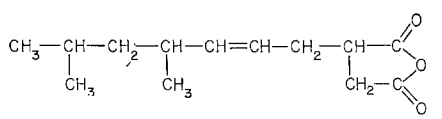
D.E.R. 736 is also compatible with the other components in the medium, and as a reactive diepoxide it becomes cross-linked in the cured system and therefore an integral part of the polymer structure. It is regarded as a flexible epoxy resin by virtue of its long aliphatic structure derived from the condensation of polypropylene glycol and epichlorohydrin. Its theoretical structure is:



D.E.R. 736 has an epoxide equivalent weight of 175–205 and an average molecular weight of about 380. Its specific gravity of 1.14 at 25°C is sufficiently close to that of the other components in the medium so that it does not tend to separate out significantly during the relatively short polymerization schedules. It was selected over D.E.R. 732 because of its lower viscosity and more rapid curing properties. In some formulations and curing schedules, D.E.R. 736 actually enhances the cure over formulations having little or none of it. Since it is a shorter molecule than D.E.R. 732, it very likely penetrates tissue more readily, and results in a higher degree of cross-linking. D.E.R. 732 was used by Erlandson (*3*) as a flexibilizer for Maraglas. Variations in the amount of D.E.R. 736 provide a wide range of useful modifications of the medium as indicated in Table I. D.E.R. 736 may cause mild skin sensitization and should be handled with care.

Anhydride hardener

Nonenyl succinic anhydride, NSA, is one of the branched alkenyl succinic anhydrides based on tripropylene. It has a molecular weight of 224, and its general structural formula is:



Nonenyl succinic anhydride was selected over the widely used dodecenyl succinic anhydride (DDSA, 290 cP) and Nadic methyl anhydride (NMA, 175–275 cP) largely because of its lower viscosity (117 cP at 25.0°C, Brookfield model RVT, spindle No. 2 at 50 rpm). It is essentially water-white in color (<0.5 on the ACS scale); and, partially, for this reason, the castings have lower color than those in which DDSA is used. NSA has a specific gravity of 1.01.

In determining the amount of anhydride needed in relation to the epoxy resins used, consideration was given to the ratios commonly used in industrial applications. Skeist (*15*) reports variations of 0.6:1 to 1:1. In the medium described here, the interrelationships of the two competing epoxy resin systems, particularly as modified by an accelerator, are not known. By disregarding the flexibilizer, however, some indication that the standard formulation is within the acceptable range can be obtained. On this basis, a ratio of 0.88 anhydride

TABLE I

SUGGESTED MODIFICATIONS OF THE MEDIUM

Ingredient	Standard medium A Firm	Modifications B Hard	C Soft	D Rapid Cure	E Longer Pot Life[a] Lower Viscosity[a]
ERL-4206	10.0	10.0	10.0	10.0	10.0
D.E.R. 736	6.0	4.0	8.0	6.0	6.0
NSA	26.0	26.0	26.0	26.0	26.0
S-1	0.4	0.4	0.4	1.0	0.2
Cure schedule (hr)[b] at 70°C	8	8	8	3	16
Pot life[c] (days)	3–4	3–4	3–4	2	7

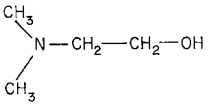
[a] As compared with standard medium A.
[b] Cure for minimum hours indicated or longer.
[c] Time between initial mixing and end point for convenient use.

to 1 of epoxide was used. Adjustments in the amount of the anhydride do not appear to be required within the range of modifications suggested in Table I.

The container with NSA should be handled in such a manner as to minimize exposure of the anhydride to water vapor in the air. NSA is regarded as nontoxic.

Accelerator

Dimethylaminoethanol, S-1 or DMAE, is one of the alkyl alkanol amines, and has the following structure:



S-1 provides typical tertiary amine cures in epoxy resin systems. It is a clear, mobile liquid with a combining weight of 89–93, a viscosity of 3.32 cP at 25°C, and a specific gravity of 0.89 at 20°C. S-1 was selected over benzyldimethylamine (BDMA), tris(dimethylaminomethyl)phenol (DMP-30), and other accelerators because it gives a longer useful pot life (3–7 days) to the embedding medium and results in castings with lower color. In addition it induces a rapid cure when the temperature is elevated to 70°C. It is also effective in low concentrations (about 1.0 % of the total resinous materials). It induces more rapid cures and results in castings of lower color than when diethylaminoethanol (S-2 or DEAE) is used. Although not regarded as hazardous, it may cause local irritation and should be handled with the precautions applied to any caustic agent.

STANDARD EMBEDDING MEDIUM

A wide range of formulations was tested with a variety of plant materials. Because such materials are characterized by tough, and sometimes hard, lignified cell walls, a formulation which yields castings that are quite firm was selected for general use.

ERL 4206	10.0 g
D.E.R. 736	6.0 g
Nonenyl succinic anhydride (NSA)	26.0 g
S-1	0.4 g
Total	42.4 g

SOURCES OF MATERIALS

ERL-4206—Union Carbide Corp., 270 Park Ave., New York, New York 10017; Union Carbide International Co., 270 Park Ave., New York, New York 10017. This material was formerly designated UNOX Epoxide 206.

D.E.R. 736—The Dow Chemical Co., Plastic Sales, Midland, Michigan 48640; European headquarters: Dow Chemical Europe–S. A., Alfred Escher-Strasse 39, 8027 Zurich, Switzerland.

Nonenyl succinic anhydride (NSA)—The Humphrey Chemical Co., Devine St., North Haven, Connecticut 06473. Specify NSA *specially refined for electron microscopy*.

Expoxy curing agent S-1—Pennsalt Chemical Corp., Three Penn Center, Philadelphia, Pennsylvania 19102; European representative: Fabriek van Chemische Producten, Vondelingenplaat N. V., P. O. Box 7120, Rotterdam, Holland.

PROCEDURE

Preparation

Gravimetric methods have been found more precise than volumetric methods in measuring viscous materials. Add each component of the medium *in turn* to a container such as an Erlenmeyer flask. Care must be used in dispensing the final amount of each component so that no excess is added. A medicine dropper pipette is convenient for this. Polyethylene dispensing bottles or burettes may be used for the first two resinous components, the ERL-4206 and the D.E.R. 736, but not for the anhydride. After adding the D.E.R. 736 and the NSA, swirl or gently agitate the mixture. Mix thoroughly after addition of the S-1 by shaking briefly, but this should be done so as to minimize entrainment of air. A motor-driven stirrer is not necessary. Bubbles incorporated during mixing readily dissipate, but this can be facilitated by the application of a light vacuum. This may also help remove any dissolved gases in the medium. The final mixture, with accelerator, can be used immediately for infiltration. The remaining medium not used for infiltration can be used for the castings. Use of the complete medium from the outset of infiltration avoids the problem, sometimes encountered, of incomplete polymerization in the center of tissue blocks when the accelerator is added to the mixture in the final change, as in some Araldite procedures. If desired, however, the accelerator can be omitted until the final change of infiltration.

Although the medium remains fluid for several days, permitting considerable

latitude in the timing of its use, holding the medium for long periods at ambient laboratory temperatures is not recommended as partial polymerization may interfere with infiltration. The medium becomes solid when held in a deep freezer and after thawing can be polymerized as usual. The medium should be protected from dust and from excessive exposure to water vapor in the air.

Dehydration

The embedding mixture is compatible in all proportions with the following dehydrating fluids: acetone, dioxan, ethanol, hexylene glycol, isopropyl alcohol, propylene oxide, tert.-butyl alcohol.

In our laboratory dehydration is usually through a graded series of ethanol (20, 40, 60, 95, 100 %), 30 min in each, with an additional change in the 100 %. The schedule can be materially shortened or altered according to the wishes of the investigator. Use a minimal volume on the last change in 100 % to avoid use of an excessive amount of embedding mixture in the next step of infiltration. Dehydration is usually done at room temperature; however, there is some indication that refrigeration may help to minimize the loss of some constituents from the specimens. It should be emphasized that the medium is completely compatible with ethanol. This is in contrast to some epoxy resin mixtures (*3*, *7*, *12*) that are not compatible with ethanol in all proportions and require the use of propylene oxide following ethanol dehydration.

To help avoid loss of lipids, a graded series of hexylene glycol and water (10, 20, 40, 60, 80 %, and two changes of 100 %) has been used occasionally for dehydration with success (Figs. 1 and 2). When this glycol is used, the pieces of tissue should be small, and the duration of infiltration should be increased to ensure adequate removal of the hexylene glycol. The medium is not compatible in all proportions with lower molecular weight glycols such as ethylene glycol, diethylene glycol, and propylene glycol.

If propylene oxide is selected as the dehydrating fluid, its incomplete miscibility with water in some mixtures above approximately 40 % can be overcome by the addition of a small amount of ethanol. About 2.5 ml of ethanol per 100 ml of propylene oxide and water will effect miscibility. This avoids the use of two dehydrating fluids, ethanol followed by propylene oxide, as recommended by Luft (*12*).

In addition to the dehydrating fluids mentioned above, the medium is compatible with several common reagents such as xylene, petroleum ether (pentanes and hexanes), and *n*-butyl alcohol.

Infiltration

Add a quantity of embedding medium that is equal to the amount of dehydrating fluid retained in the vials at the last change. Swirl the mixture and let stand for one-

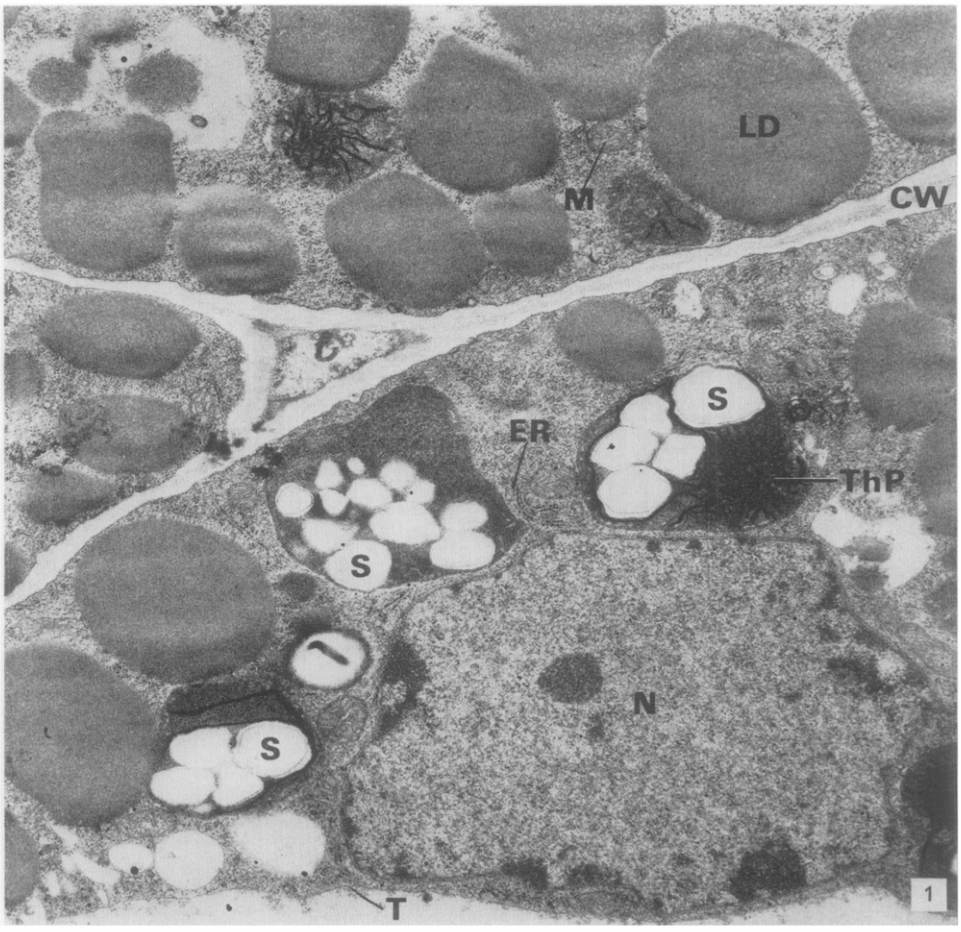


FIG. 1. Portions of three cells from tomato endosperm 6 days after initiation of germination. Observe that the lipid droplets (*LD*) are well preserved. Note plastids with several starch grains (*S*) and one with a large thylakoid plexus (*ThP*) or prolamellar body. *CW*, cell wall; *T*, tonoplast; *M*, mitochondria; *N*, nucleus; *ER*, endoplasmic reticulum with bound ribosomes. Fixation—glutaraldehyde and Dalton's osmium; hexylene glycol dehydration; uranyl acetate and lead citrate contrasting. × 10,888.

half hour. Then add another equal quantity of embedding medium to the mixture of dehydrating fluid and embedding medium in the vials. Swirl and let stand for one-half hour. Pour and drain all the mixture from the vials, add a third amount of embedding medium, and swirl again. The specimens are now in 100 % embedding medium and may be held in this, at the discretion of the investigator, until infiltration is regarded as complete. In our laboratory relatively large or dense pieces of material

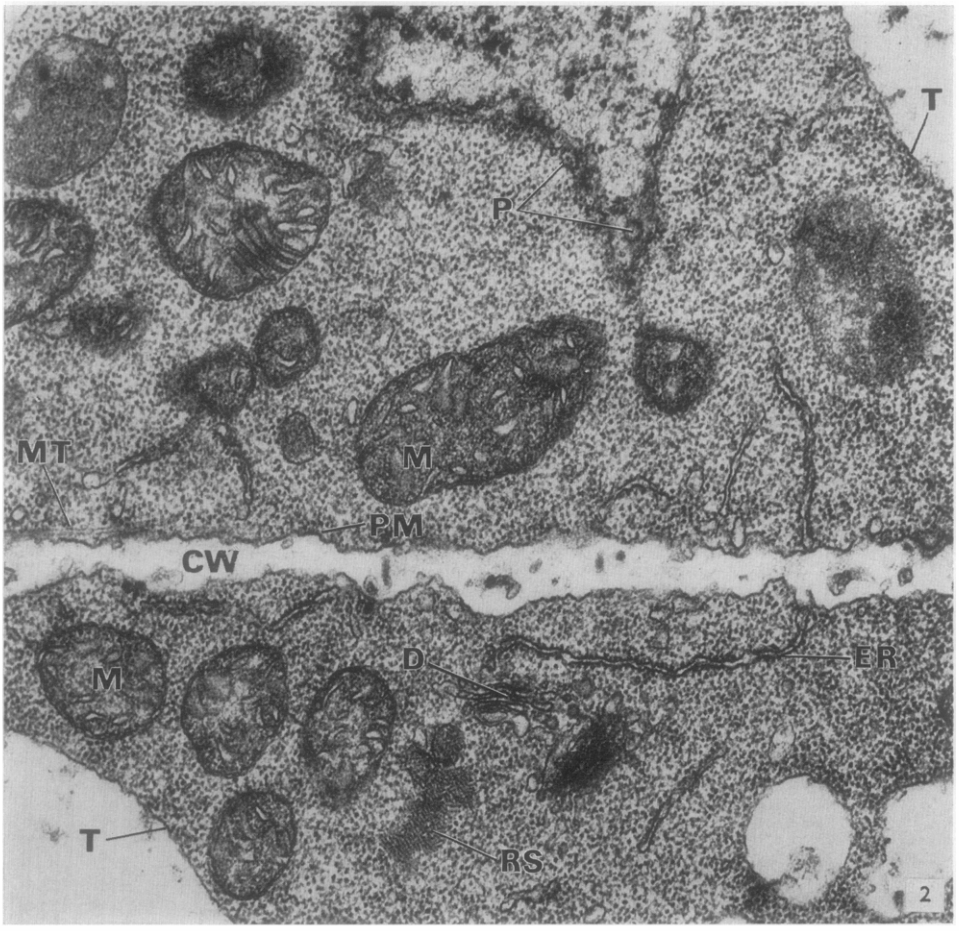


FIG. 2. Portions of two cells in the root tip of an 8-day-old tomato seedling. Observe ribosomes free and bound to the endoplasmic reticulum (*ER*) and a portion of a nuclear envelope with pores (*P*) in surface view. *CW*, cell wall; *PM*, plasma membrane; *T*, tonoplast; *M*, mitochondria; *MT*, microtubule; *D*, dictyosome; *RS*, reticulate structure. Fixation—glutaraldehyde and Dalton's osmium; hexylene glycol dehydration; uranyl acetate and lead citrate contrasting. × 34,400.

are infiltrated during the afternoon and left overnight in a second change of 100 % plastic. With small pieces a convenient schedule is to infiltrate during the afternoon and polymerize overnight (ca. 16 hours). Success has also been achieved with many specimens by going directly into 100 % embedding medium at the second step in infiltration and then repeating again with 100 % plastic in the third change. Continuous mild agitation during infiltration is provided by attaching the vials to a rotating (ca. 1 rpm) motor-driven wheel (25 cm diameter inclined at 45 degrees).

Embedding and polymerization

To embed, fill *oven-dry* gelatin capsules or other embedding forms with the embedding medium (from the same batch used for infiltration) and transfer the specimens to them with a medicine dropper pipette, applicator stick, forceps, or dissecting needle. The specimens will generally sink to the bottom of the embedding medium. Polymerize in an oven at 70°C for 8 or more hours. Curing schedules longer than 8 hours for the standard medium do not harm the castings, and the cure may improve with longer schedules. The standard medium may be cured in 6 or 7 hours if the interval between preparation of the medium and initiation of the oven cure is over 24 hours. Polymerization is apparently quite uniform throughout the castings, and during this process there is apparently no significant shrinkage that would alter the specimens or interfere with their sectioning. Furthermore, when polymerized adequately the castings do not shrink noticeably on cooling after removal from the oven. The castings are transparent and water-white to light yellow in color. When polymerization takes place under conditions that exclude air entirely, and possibly water vapor, the castings may develop a very light pinkish tint; but this does not detract from their cutting quality. This condition can be prevented by adding diethylene or hexylene glycol to the mixture. A most noticeable effect on the quality of the casting occurs when gelatin capsules are placed in wooden holders that have not been oven-dried immediately prior to use. Water vapor from the wood makes brittle the basal portion of the casting and may delay polymerization to the extent that, after removal from the oven, bubbles may form in this portion of the casting. Precautions must be taken to avoid excessive exposure to moisture of the medium and the materials, especially the acid anhydride, before use. However, removal of the gelatin capsules by tap water does not make the castings tacky or noticeably change their physical properties.

Trimming and some physical properties of the castings

The castings trim well and are not prone to gross fracturing or shattering as were some experimental formulations in this study. Reedy (*13*) also reported fracturing in systems containing hexahydrophthalic anhydride (HHPA) or Nadic methyl anhydride (NMA). The deflection temperature (DT) of the embedding medium was determined on 5 of 8 bars, $6 \times 1/2 \times 1/2$ inches, cast in a steel mold and cured 24 hours at 65°C (American Society for testing materials method D648-56, 4-inch span). The average DT was 45.0°C. This is at the lower end of the range (40–150°C) reported by Skeist (*15*) for many industrial applications of epoxy resins. Should castings with a distinctly higher DT be prepared by lowering the amount of D.E.R. 736, brittleness while trimming may be overcome by shining a strong spotlight on the casting.

Sectioning

In this study sections were prepared with a Cambridge ultramicrotome using glass knives or a Ge-Fe-Ri diamond knife mounted in a Wesfall-Healy section mounter. The block face is quite hydrophobic; consequently, it does not wet readily from the distilled water in the trough during sectioning. Solutions of ethanol or acetone are not recommended for use in the trough, as they may wet the block face more readily because of their lower surface tensions. If they are required to achieve adequate wetting of the knife edge, the lowest effective concentrations should be used. The sections flatten well on the surface of the distilled water in the trough, form intact ribbons and do not noticeably compress or expand. A critical evaluation of these latter characteristics, however, was not made. With favorable tissues, sections of relatively large area can be prepared. Thicker monitor sections may also be prepared for light microscopy.

The sections are tough under the electron beam and can be routinely used on 200-mesh grids without a supporting membrane. The temperatures induced by a high-intensity electron beam are presumed to be somewhat higher than the oven temperature (70°C) used in the cure. This may enhance the cure in localized areas of the sections on which the beam impinges, as many epoxy resin systems are reported by Skeist (*15*) to postcure best at a temperature considerably higher than the curing temperature. Although the methods used by Cosslett (*2*) to determine the effect of the electron beam on the sections were not used on this material, routine observations of the sections at 50 kV revealed no obvious indications of sublimation or other evidence of degradation.

Application and evaluation

This medium has been satisfactorily used on a wide range of tissues, including such problem materials as the lipid storage endosperm of seeds of castor bean and tomato, the mature, dry seed coats of tomato and the hard, lignified elements in the vascular bundles of tomato leaves. Without alteration the medium is also well adapted to the soft, highly vacuolated, parenchymatous tissues of ripe tomato fruits. Recently published micrographs of materials embedded in the medium or in slight variations of it, after ethanol dehydration may be examined in reports by Spurr and Harris (*17*), Appelqvist *et al.* (*1*), and Ku *et al.* (*8*). Lu (*11*) has used it on the basidiomycete *Coprinus*. It has also been used successfully on two ascomycetes, *Saccharomyces* and *Niteolla*, and on a variety of tissue fractions and pellets prepared by centrifugation. Materials which were embedded in the medium after dehydration in hexylene glycol are illustrated in Figs. 1 and 2. Here, as well as at high magnifications, there is no obvious indication of graininess in the resinous background areas. This indicates that the castings may have a very fine structure, a characteristic favored by the rela-

tively low molecular weights of all the components of the medium. It should be recognized that a number of phenomena apparently account for graininess in the photographic plates and micrographs, including, for example, phase grain, background noise in the electron microscope, and the method of processing of the photographic materials. It would be difficult at high-resolution electron microscopy to differentiate between graininess caused by these and other factors and that due to fine structure in the plastic. Globular formations, however, have been noted by Erath and Spurr (*4*) in a number of thermosetting resins, including an epoxy resin, cured by heating and by pressure molding at the semigel stage. Micellar structures on the order of 400–900 Å were demonstrated mainly by shadowing and by leaching the resins with acetone. Without using these special techniques nothing corresponding to the coarse structures that they observed were seen in the medium described here. Lee and Neville (*10*) comment on evidence indicating the formation of three-dimensional agglomerates in cured epoxy resins. They also suggest that resins with low viscosity may develop better cross-linked agglomerates than resins of high viscosity because of enhanced molecular mobility during the curing process. If this is the case for the low viscosity medium described there, especially in view of the high reactivity of the ERL-4206 and the moderately rapid cure provided at elevated temperatures by the accelerator, S-1, the castings probably have a very fine order of structure.

Like many cured epoxy resins, the castings are relatively inert and have excellent chemical resistance. They are particularly resistant to strong oxidizing agents. Accordingly, grid staining of the sections with such materials as $KMnO_4$ or $Ba(MnO_4)_2$ has no noticeable effect on the electron transmission of the background plastic. This is an improvement over an Epon 812 epoxy resin system (*12*) containing nadic methyl anhydride (NMA), since the cured system is highly subject to oxidation, as observed in this study and as reported by Reedy (*13*). Apparently the double bond at carbon 2 in nonenyl succinic anhydride in the cured system is relatively resistant to oxidation. The common fixation techniques using glutaraldehyde, osmium tetroxide, and the permanganates, and the techniques for enhancing contrast using materials such as uranyl acetate and lead citrate are compatible and have given good results with this medium. Grid stains seem to penetrate the plastic readily, or at least enter by way of the exposed organic material at the section surface. Tissue sections have not been evaluated with ultrastructural cytochemical tests. The effect of the medium or of its separate resinous components as an extractant of tissue materials before polymerization was not investigated. Its low viscosity, however, would permit infiltration under refrigeration, which might minimize the loss of some constituents. The physical properties and cutting quality of the castings do not change noticeably under prolonged storage at room temperature. Excellent results have been obtained in staining monitor sections for light microscopy with the Azur II-methylene blue method of Richardson, Jarett and Finke (*14*).

Modifications of the medium

To achieve special characteristics in the medium or the castings, some suggested modifications of the standard medium are summarized in Table I. These give some indication of the kinds of modifications that are feasible. All the modifications have been tested successfully with mature tomato leaf tissue. Modifications favoring one feature generally involve sacrifice of other qualities of the medium. Thus, for example, for a medium that features maximum pot life and minimum viscosity (Table I, E mixture) through a reduction of the accelerator S-1, a longer polymerization schedule is required. Or, if very short cure schedules are desired, by increasing the accelerator (Table I, D mixture) the pot life is reduced, and the viscosity is increased. This does not seriously affect the use of the medium providing rapid infiltration schedules are followed shortly after preparing the medium. Curing may also be accomplished at temperatures lower or higher than 70°C. Higher temperatures will accelerate polymerization and result in a more complete cure, but tissue damage may be encountered; there may also be more shrinkage that can be attributed to cooling after removal of the castings from the oven. Nonreactive diluents such as dibutyl phthalate may be added to the medium providing the amount of D.E.R. 736 flexibilizer in the medium is reduced. Modifications other than those suggested may be found desirable for general use or for special plant or animal tissues. Once a mixture has been adequately cured there is great latitude in the time of removal of the castings from the oven. This is in contrast to the brief time margins reported for some epoxy resin systems (*14*) between a soft casting and one that is too brittle.

The low-viscosity ERL medium described here provides several distinct advances over other epoxy resin formulations commonly in use. Although much remains to be developed about the medium as well as other embedding methods in reference to their effects on specimens, this method and its modifications are recommended for wide application and evaluation with plant and animal materials.

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