IMPROVEMENTS IN EPOXY RESIN EMBEDDING METHODS

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ABSTRACT

Epoxy embedding methods of Glauert and Kushida have been modified so as to yield rapid, reproducible, and convenient embedding methods for electron microscopy. The sections are robust and tissue damage is less than with methacrylate embedding.

It has become apparent that there is considerable advantage in using epoxy resins in embedding tissues for electron microscopy. Compared to methacrylates, epoxy resins offer remarkable freedom from polymerization damage, with consequent excellent preservation of cellular fine structure and intercellular relationships (Birbeck and Mercer, 1956). Epoxy sections also seem to suffer less degradation during irradiation by the electron beam than do those cut from methacrylate. They can be used with many fixatives without danger of "bubbling."

The first practical method of embedding in epoxy resins was that of Glauert *et al.* (1956, 1958). This procedure has by now received a considerable trial, and has yielded very fine results in the hands of some investigators.

On the other hand, we have had difficulty in using these resins, even with the British materials[1] said to be identical to those recommended by Glauert *et al.* (1956, 1958). The use of so called "equivalent" materials of United States origin has given even more trouble. Problems encountered with these Araldites involve (1) inadequate penetration of tissue blocks (1 to 1.5 mm. cubes) in the recommended times, and (2) difficulties in cutting, resulting in corrugated or shattered sections, even after prolonged soaking in the liquid resin in attempts to achieve adequate impregnation.

These difficulties have been apparent in other laboratories as well, and have led to the development of embedding methods based on other epoxy resins. Thus Kushida (1959) and Finck (1960) have published methods using an aliphatic epoxy resin marketed by the Shell Chemical Corporation under the name, Epon 812 (previously known as Epon 562). These methods have not been tried in this laboratory.

Quite independently, over the last three or four years, there have been developed in this laboratory two separate approaches to epoxy embedding. These have had sufficient success to warrant publication. The first approach involved use of the aromatic Araldite resins of the Ciba Company, adapting these materials to yield rapid, reliable procedures of embedding. The second approach was similar in some respects to that of Kushida (1959) and Finck (1960). It is likewise based on aliphatic Epon 812 and has led to rapid, flexible, versatile, and reliable methods of embedding which have become routine in this laboratory. These Epon methods have been sent to several laboratories in other cities, where they have been applied successfully. They are now used in our laboratories for instruction of beginners in biological electron microscopy. Students beginning with Epon embedding methods have, in

[1] The author is indebted to Dr. H. Huxley, University College London, to Professor J. T. Randall, King's College, London, to Dr. M. S. C. Birbeck, Chester Beatty Research Institute, London, and to Mr. H. Voorhees, Ciba, Inc., Kimberton, Pennsylvania, for gifts of the British materials; *i.e.*, Araldite casting resin M, hardener 964B, and accelerator 964C.

our experience, achieved satisfactory specimens in a much shorter time than was necessary when methacrylate was used. Methacrylate has been abandoned in our laboratories as an embedding medium for electron microscopy.

The new Araldite and Epon procedures have led to significant improvements in case of penetration and of cutting, as compared to Glauert and Glauert's (1958) Araldite methods. For most tissues, the new methods permit embedding of tissue cubes from fixation to sectioning in 24 to 48 hours. The blocks can be cut nearly as easily as can the methacrylates. With the Epon resin the hardness can be readily varied over a wide range. In the electron microscope, Epon sections yield better contrast than Araldite preparations. Either Epon or Araldite sections can be stained readily, either on the grid for electron microscopy, or as 1 μ sections for light microscopy (Heidenhain's iron-hematoxylin). The sections are robust and often can be examined in the electron microscope without any supporting film at all, or on a simple carbon film. Sandwiching techniques (Watson, 1957) are unnecessary. Epon materials seem to be easily available inside or outside North America without the difficulties attendant to procurement of the Ciba Araldites. These developments were expedited by the excellent book on epoxy resin technology by Lee and Neville (1957).

MATERIALS

The modifications reported in this paper utilize the following materials, in addition to those commonly used in laboratories engaged in sectioning for electron microscopy.

Araldite 502—Ciba Company, Inc., Plastics Division, Kimberton, Pennsylvania.

Dodecenyl succinic anhydride (DDSA) (hardener)—National Aniline Division of Allied Chemical and Dye Corp., New York City.

Epon 812—Shell Chemical Co., San Francisco.

Methyl nadic anhydride (MNA) (hardener)—National Aniline Division of Allied Chemical and Dye Corp., New York City.

2,4,6-tri (dimethylaminomethyl) phenol (DMP-30) (accelerator)—Rohm and Haas Co., Philadelphia. Eight ounce bottles available as free sample on request.

Propylene oxide (1-2-epoxy propane)—Matheson Coleman and Bell, Norwood (Cincinnati), Ohio; East Rutherford, New Jersey; also Eastman Organic Chemicals, No. 2068, Distillation Products Industries, Rochester, New York.

MODIFIED "ARALDITE" METHOD

The main defect of Glauert's Araldite, that of slow penetration, was ameliorated by using propylene oxide as an additional stage in the transition between the dehydrating alcohols and the liquid resin. Various reactive diluents are available to reduce the high viscosity of the liquid epoxy resin mixture, but most of these tend to degrade the mechanical properties of the cured, solid resins. (Non-reactive diluents, such as xylene, etc., are even worse in this respect and may even separate from the resin during polymerization). A series of mono-epoxides was tried as reactive diluents at the ratio of 10 per cent v/v in the final resin. The mono-epoxides used in these trials were C_{16}-C_{18} olefin oxide, dodecene oxide, dipentene monoxide, octylene oxide, butylene oxide, and propylene oxide.[2] This series revealed that the long-chain mono-epoxides seriously detracted from the cutting qualities of the cured block, producing materials with properties resembling an art-gum eraser in extreme cases. However, the short-chain members (butylene oxide and propylene oxide) gave cured blocks which cut fairly well, but no better than the undiluted resin alone. Propylene oxide was the best of the series. It has very low viscosity and a low boiling point (similar to ethyl ether in both respects), is quite soluble in water, and miscible in all proportions with alcohol and epoxy resins. It quickly replaces most of the alcohol used for dehydration and rapidly diffuses out of the tissue block to be replaced by the liquid resin. Traces remaining within the block cause no difficulty. Although no advantage results, propylene oxide can be used in transition between alcohol and methacrylates. This does, however, reveal that the propylene oxide step causes no detectable damage or additional extraction over the usual methods of methacrylate embedding.

This statement must be qualified if the tissue has been stained first in the alcohol stages with phosphotungstic acid and subsequently embedded

[2] The C_{16}-C_{18} olefin oxide, dodecene oxide, dipentene monoxide, and octylene oxide were generously provided by Dr. R. V. Gall of the Becco Chemical Division of the Food Machinery and Chemical Corp., Buffalo, New York. The author is grateful to Dr. Henry Lee of the Epoxylite Corp., El Monte, California, for information on the sources of these diluents and for other information about specific resins.

in the highly reactive Epon resin. In this case, considerable extraction of the PTA occurs. Acids in general, and PTA in particular, are reactive with epoxides (Lee and Neville, 1957); in the case of solid PTA and pure propylene oxide, the reaction is explosive. Staining of the tissue block can be preserved by removing the excess protons from the bound PTA with alkali, as follows: Following the usual PTA treatment and a 30-minute absolute alcohol rinse, the tissue blocks can be exposed for 15 to 30 minutes to 0.01 per cent NaOH in absolute alcohol followed by a 30-minute rinse in two changes of absolute alcohol. The tissue may then be run into propylene oxide and embedded by one of the methods described here.

The second problem, namely difficulty in sectioning, has been lessened by two alterations in the usual procedure: (1) by modifying the temperature at which the resin is cured, and (2) by selecting a different resin proportion.

Unpublished experiments by the author using methacrylates indicate that difficulty in sectioning seems to be correlated more with the degree of cross-linkage of the plastic than with hardness of the block. Thus, using a Porter-Blum microtome and a glass knife, *n*-butyl methacrylate polymer becomes more and more difficult to cut as the plastic is cross-linked chemically more and more heavily. This cross-linkage is easily varied in a controlled manner by adding varying small amounts of agents such as ethylene glycol dimethacrylate to the monomer before polymerization. The greater the amount of difunctional methacrylate present, the greater the degree of cross-linkage. The degree of cross-linkage can be assayed by solubility and swelling tests. Conversely, the poly-*n*-butyl methacrylate blocks can be cut more and more easily and with duller knives as chain-transfer agents (Riddle, 1954) are added to the monomer before polymerization. These agents, such as benzene thiol or 2-naphthalene thiol, tend to reduce the average molecular weight of the polymer chains and narrow the distribution of chain lengths, producing a molecularly more uniform polymer of sharper melting point. (Unfortunately, polymerization damage was also worse in the presence of these chain-transfer agents). These relationships of cross-linkage and molecular weight to section cutting are consistent with the hypothesis that cutting of these resins represents a zone-melting at the knife edge and a parting of the section from the block at the advancing molten or softened region.

Many of the attractive features of epoxy resins for industrial applications result from the mechanical properties associated with a high degree of cross-linkage, which can be obtained with the formulations recommended for commercial use. These highly cross-linked resins prove to be very difficult to cut. Epoxy resins cured by monoanhydrides are chemically less subject to such cross-linkage than are resins cured by other means. Moreover, according to Lee and Neville (1957), the amount of cross-linkage can be modified further to some extent by varying the temperature of the curing cycle. Thus low temperatures are said to favor a linear polymer with few cross-linkages. Lee and Neville state further that once the molecular architecture of the gelled resin is established, it is less likely to be influenced by subsequent higher temperatures, which are, nevertheless, necessary to increase the degree of cure. For purposes of tissue embedding, a cool initial incubation period might be useful to evaporate off the propylene oxide without boiling (b.p. 35–37°C.).

In view of the arguments presented by Lee and Neville (1957), a three-stage curing program was chosen with incubation at 35°, 45°, and 60°C. for about 12 hours at each temperature. The resulting resin has a low heat distortion point, indicating a low degree of cross-linkage. The combination of propylene oxide and this three-stage cure made it possible to use the United States equivalent of Araldite casting resin M, namely Araldite 502, in a rapid modification of the procedure described by Glauert *et al.* (1958). As used here less than 48 hours elapsed between embedding and finish of cure. Good rapid results were obtained with three separate batches of Araldite 502 as well as with the British casting resin M.

Dr. R. L. Wood of this department has introduced a further significant improvement in the Araldite method by setting the anhydride-epoxy ratio at 0.7. This can be achieved by employing the following mixture:

Araldite 502	27 ml.
Dodecenyl succinic anhydride (DDSA)	23 ml.

1.5 to 2.0% *v/v* DMP-30 (accelerator) is added just before use.

The anhydride and resin are mixed very thoroughly. The mixtures are stable and usable for

several weeks if kept in the refrigerator. Precautions are taken to prevent condensation of moisture in the bottle or in the resin. Risk of contamination with water can be reduced if the bottle is allowed to warm to room temperature before it is opened, and if it is allowed to remain open only a short time.

PRECIS OF *ARALDITE* PROCEDURE

1. The tissues are fixed and dehydrated with ethyl alcohol. One change in absolute alcohol is adequate.

2. The absolute alcohol is poured off. The tissue blocks are immersed in two changes of propylene oxide for 10 to 15 minutes each. Decantation and refilling is done quickly so that the tissue does not dry out on the surface. The propylene oxide is flushed down the sink drain with cold water. Precautions are taken to avoid evaporation of the liquid and inhalation of the vapors.

3. The second change of propylene oxide is poured off and 1 to 2 ml. of fresh propylene oxide is added. An equal quantity of the freshly prepared resin mixture as recommended by Wood (see above) containing the accelerator (1.5 to 2.0 per cent) is added and mixed by swirling. After 1 hour another equivalent volume of the resin mixture is added. Three to 6 hours are allowed for infiltration. The longer period of time is preferred if time permits.

4. The required number of capsules are filled to within 1 to 2 mm. of the top with the resin mixture containing 1.5 to 2.0 per cent accelerator (DMP-30) but no propylene oxide. The specimens are taken up in a large bore pipette. The specimen is allowed to settle to the tip of the pipette and transferred to the capsule with a minimum of propylene oxide mixture. The tissue block is sometimes placed on a wooden tongue blade for draining excess propylene oxide mixture and then introduced to the capsule by touching the block to the resin surface. It is not necessary to stir the resin in the capsule after introducing the specimen.

5. The resin in the capsules is then polymerized according to the following schedule:

(*a*) Overnight at 35°C.
(*b*) Next day at 45°C.
(*c*) Next night at 60°C. Can be sectioned the next day.

Satisfactory results also have been obtained by placing the capsules directly into an oven at 60°C. for 12 hours.

It is deemed important to allow sufficient infiltration time (3 to 6 hours), to measure the accelerator fairly accurately and to mix the resin and accelerator thoroughly. The 35°–45°–60° oven sequence for polymerization is preferred. Sections may be cut directly onto distilled water. The block should trim and cut in a manner comparable to the 50:50 DDSA-MNA Epon mixture (see below). There is some clearing under the beam but the contrast at the microscope is less than that with Epon embedding. The tissues are preserved very well, with perhaps some differences from Epon. Araldite sections may be stained with heavy metals in the same manner as Epon and the sections can be self-supporting on a 200-mesh or 300-mesh grid with somewhat less difficulty. Araldite embedding appears to preserve relationships in connective tissue regions with less distortion than is sometimes seen with Epon.

EPON EMBEDDING METHOD

A resin combination using Shell Epon 812 allows for even more rapid penetration. Epon 812 is a glycerol-based aliphatic epoxy resin of low viscosity. Blocks prepared by our method cut rather easily. The sections show greater contrast in the electron microscope than do comparable Araldite sections. With this resin it is possible to have sufficient contrast to permit use of osmium tetroxide fixation alone without additional staining.

When dodecenyl succinic anhydride (DDSA) is used alone as the curing agent (as in the Araldite procedure), the blocks based on Epon 812 are rather soft. Another liquid anhydride, methyl nadic anhydride (MNA or methyl endomethylene tetrahydrophthalic anhydride), produces much harder blocks. The two can be mixed in any proportion to give a continuous and wide range of hardness. This feature is used to give a two-mixture system with flexibility and convenience. The anhydride:epoxy (A:E) ratio of the mixtures is reduced from the value of 0.9 to 1.0, recommended for industrial use of Epon 812, to 0.70. This gives considerable improvement in cutting quality.

PRECIS OF *EPON* METHOD

1. The tissue is fixed as usual and dehydrated up through absolute ethanol.

2. The blocks are put through two successive changes of propylene oxide for 15 to 30 minutes each. This stage can be omitted.

3. The last change of propylene oxide or absolute alcohol is poured off and 2 to 3 ml. fresh solvent is

added. An estimated equal quantity of the complete mixed resin (containing accelerator) is then added and mixed by swirling. This is allowed to remain at least 1 hour, swirling occasionally.

4. Gelatin capsules are filled nearly full with the complete resin mixture (see below, under 6). Each tissue block from stage 3 is sucked up into a pipette, allowed to settle to the tip, and transferred with a minimum of the liquid to the surface of the resin in the capsule. The tissue block slowly settles to the bottom of the capsule, losing most of its solvent on the way. It is not necessary to mix or stir. (This is adequate for No. 00 capsules. With smaller capsules or very large tissue blocks, it is better to allow the tissue blocks to settle first through 5 to 10 ml. of the complete resin in a beaker and then transfer them to the capsules).

5. The resin is cured overnight at 60°C., or subjected to the following polymerization schedule:

(*a*) Incubation overnight at 35°C.

(*b*) Incubation next day at 45°C.

(*c*) Incubation next night at 60°C.

The blocks can be cut on the morning following completion of polymerization. However, the ease of cutting improves with age. A day at each stage in the oven may improve the cutting quality. Under mild conditions such as these, the cure is always far from complete. Even under drastic laboratory conditions the cure never reaches completion (Lee and Neville, 1957).

6. The resin components are made up as follows:

Mixture A	
Epon 812	62 ml.
DDSA	100 ml.
Mixture B	
Epon 812	100 ml.
MNA	89 ml.

These are all moderately viscous liquids. They should be thoroughly mixed. Under refrigeration they have a life of up to 6 months, but several days at room temperature does no harm. The components need not be dried, but can be used as obtained from the manufacturer. However, they should be protected from water, and if the mixtures are stored in the cold, they should be warmed past the dew point before opening. The proportions are chosen to give an anhydride:epoxy ratio of 0.70. This is generally not optimum for industrial use, but seems to give satisfactory results for sectioning.

Immediately before use, the accelerator, DMP-30 (2,4,6-tri(dimethylaminomethyl)phenol), is added to the selected resin mixture in the proportion of 1.5 to 2.0 per cent *v/v*, and stirred *thoroughly*. This constitutes the *complete resin mixture* referred to above.

Most failures seem to arise from failure to mix adequately these viscous materials. It is convenient to measure the resin mixtures into a 10 to 15 ml. conical pharmaceutical graduate (Kimble 60340), stirring for a full 5 minutes, continually sweeping out the entire volume of the graduate with the stirring rod. Mixtures *A* and *B* pour easily. The small quantities of DMP-30 should be accurately measured and delivered. This can be done conveniently with a tuberculin syringe with a long, large bore needle. The propylene oxide can also be conveniently dispensed with a 10 ml. syringe and long needle, thus avoiding excessive exposure to the vapor of this moderately toxic agent. The DMP-30 can cause a severe contact dermatitis unless handled carefully (Lee and Neville, 1957).

The hardness of the final resin depends upon the ratio of *A* to *B* in the complete resin mixture. *A* alone gives a soft block, whereas *B* alone is very hard. An approximate hardness scale (estimated by needle penetration) of the following mixture is included in Table I.

The procedure outlined here has similarities to methods developed independently and recently published by Kushida (1959), in which hardness of the final block is adjusted by varying proportions of Epon 815 and Epon 562 (now called Epon 812), both of which are combined with dodecenyl succinic anhydride, and to a method of Finck

TABLE I

Mixture A (volume)	Mixture B (volume)	Total volume resin	Accelerator (DMP-30) (volume)	Equivalent hardness as related to methacrylates
(*ml.*)	(*ml.*)	(*ml.*)	(*ml.*)	
10	0	10	0.15	100% *n*-butyl meth.
7	3	10	0.15	10–15% methyl meth. in *n*-butyl
5	5	10	0.15	15–20% methyl meth. in *n*-butyl
3	7	10	0.15	20–30% methyl meth. in *n*-butyl
0	10	10	0.15	30–50% methyl meth. in *n*-butyl

(1960) in which a reactive plasticiser is used to vary the resin hardness.

SUMMARY

Epoxy resin embedding methods are presented consisting of variations of the original Araldite procedure of Glauert *et al.* (1958). The advantages of the methods are (*a*) rapid embedding, (*b*) easy sectioning of the embedded tissue, (*c*) good contrast in the electron microscope, and (*d*) a wide range of hardness. Infiltration is accelerated by using propylene oxide after the alcohol dehydration. Easier sectioning results from a gradual cure through increasing temperatures and a low anhydride: epoxy ratio in the resin. A wide hardness range can be achieved by using two different anhydride curing agents.

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