Epoxy Resins in Electron Microscopy*

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PLATES 5 TO 9

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ABSTRACT

A method of embedding biological specimens in araldite 502 (Ciba) has been developed for materials available in the United States. Araldite-embedded tissues are suitable for electron microscopy, but the cutting qualities of the resin necessitates more than routine attention during microtomy. The rather high viscosity of araldite 502 also seems to be an unnecessary handicap.

The less viscous epoxy epon 812 (Shell) produces specimens with improved cutting qualities, and has several features—low shrinkage and absence of specimen damage during cure, minimal compression of sections, relative absence of electron beam-induced section damage, etc.—which recommends it as a routine embedding material. The hardness of the cured resin can be easily adjusted by several methods to suit the materials embedded in it.

Several problems and advantages of working with sections of epoxy resins are also discussed.

Epoxy Resins in Electron Microscopy

The technique of embedding specimens in epoxy resins for electron microscopy was introduced by Maaløe and Birch-Andersen (1), and markedly improved by Glauert and coworkers (2, 3). Although the method has been used successfully in several laboratories (Birbeck and Mercer (4); Robertson (5), it has failed in others (6). Our further modifications have been uniformly successful in this and other laboratories where they have been used with a variety of tissues prepared by several methods of fixation.[1] The newer method employs an epoxy resin of very low viscosity which can be hardened at low temperatures to produce a block with excellent cutting qualities.

The Araldite Mixture

Our initial attempts to adapt Glauert's procedure to materials available in the United States were encouraging, but not entirely satisfactory. Several trials in which proportions were varied proved the following mixture to be optimal:

Araldite 502[2] epoxy resin	10.0 ml.
Dodecenylsuccinic anhydride[3] (DDSA) hardener	10.0 ml.
Benzyldimethylamine[4] (BDMA) accelerator	0.2 ml.

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[1] After fixation by osmium tetroxide, formaldehyde, potassium permanganate, acrolein, freeze-drying, and freeze-substitution, the following tissues and organisms have been examined: liver (embryonic and adult), pancreas, parotid gland, duodenum, heart and skeletal muscle, peripheral and central nervous system (embryonic and adult), the ameba *Chaos chaos*, and *Stentor coeruleus*. The tissues were from cat, mouse, rat, guinea pig, chicken, salamander, frog, goldfish, and squid.

[2] Obtained from Ciba Products Corp., Fair Lawn, New Jersey. Pertinent information is available in *Techn. Data Bull. 4.*

[3] Dodecenylsuccinic anhydride and hexahydrophthalic anhydride were obtained from the National Aniline Division of Allied Chemical and Dye Corp., New York. The anhydrides must be protected from moisture since the free acids induce excessively rapid cures and impair the final properties of the hardened resins.

[4] Benzyldimethylamine was obtained from the Maume Chemical Co., Toledo. The amine is a severe sensitizing irritant and contact with it or its vapors should be avoided.

This mixture gels in about 18 hours at 40°C., and is suitable for microtomy after 2 to 4 days additional curing at 55°C. The resin as supplied by the manufacturer is modified with an unspecified plasticizer, and addition of dibutyl phthalate only serves to deteriorate the cutting qualities of the cured resin. The final hardness of the block can be varied somewhat, however, by altering the resin/hardener ratio or by varying the time and temperature of the initial cure. Curing of the resin can be hastened by increasing either the temperature or the concentration of accelerator.

The infiltration schedule recommended by Glauert and Glauert (3) is quite adequate, but, may, if one wishes, be shortened even for tissues as diverse as liver, muscle, and central nervous system. The following procedure works well:

Absolute alcohol/araldite mixture (50/50)	1 hr. at 40°C.
Araldite mixture less accelerator	2 hrs. at 40°C., several changes
Araldite mixture, with accelerator	2 hrs. at 40°C., several changes

The tissues are then transferred to fresh araldite mixture plus accelerator in gelatin or polyethylene capsules and polymerized overnight at 40°C.

Although this procedure produces usable specimens (Fig. 1), several disadvantages still maintain. Araldite 502 is very viscous (see Table I) and its viscosity increases rapidly after addition of the amine accelerator. More serious, perhaps, is the insufficient hardness of the final block and its low softening temperature: the polymerized resin is consequently very difficult to section with anything but the best of knives if the temperature of the surroundings is above 23°C.

These disadvantages have been overcome by employing an epoxy resin mixture based on epon 812.[5]

Features of Epon 812

Epon 812 is a light colored aliphatic based epoxy resin of relatively low viscosity (see Table I). It may be hardened at low temperatures with acid anhydrides in the presence of small amounts of an amine accelerator. When cured with hexahydrophthalic anhydride[3] (HHPA) the final product is water-white; dodecenylsuccinic anhydride imparts a pale straw color to the cured resin.

TABLE I

	Araldite 502	Epon 812
Viscosity (C.P., at 23°C.)	3,000 to 6,000	90 to 150
Epoxide equivalent[6]	250	140 to 165
Color	Pale amber	Pale straw

The increased amount of hardening agent required to cure this resin in part offsets its reduced viscosity, as compared with araldite 502. But even with the hardener added, the viscosity of the mixture is much less than that of araldite 502, and its rate of viscosity increase after addition of the amine is less than with the araldite mixture.

Various Mixtures of Epon 812

When epon 812 is hardened with hexahydrophthalic anhydride alone, the cured resin is too hard and brittle to be sectioned with a glass knife. By incorporating varying amounts of the epoxy flexibilizer cardolite NC 513,[7] however, the hardness of the resin may be adjusted to suit the specimen embedded in it. Or alternatively, the hardness may be varied by curing with a mixture of hexahydrophthalic anhydride and dodecenylsuccinic anhydride. Cardolite NC 513 imparts a pale amber color to the cured resin.

Mixture A (Table II) produces a harder resin than the araldite mixture, while mixture C is hardest and toughest. By decreasing the amount of flexibilizer added, mixture C may be made harder still.

[5] Available from the Shell Chemical Corp., Sewaren, New Jersey. Epon 812 (formerly designated epon 562) is severely irritating to the eyes and prolonged or repeated skin contact will cause destruction of tissues. Technical information is available in various bulletins published by Shell.

[6] The epoxide equivalent weight is the grams of resin containing 1 gm. equivalent of epoxide. The data are helpful in calculating stoichiometric amounts of hardening agents. For further information, see: Lee, H., and Neville, K., Epoxy Resins, New York, McGraw-Hill Book Co., Inc., 1957; and, Skeist, I., and Somerville, G. R., Epoxy Resins, New York, Reinhold Publishing Corp., 1958.

[7] Cardolite NC 513 was obtained from the Irvington Chemical Division of the Minnesota Mining and Manufacturing Co., Newark, New Jersey. It is a chemically bound flexibilizer for epoxy resins, having reactive epoxy groups in its own structure. Additional information is to be found in bulletins published by the manufacturer.

TABLE II

	A	B	C
Epon 812	10 ml.	10 ml.	10 ml.
DDSA	19 ml.	18 ml.	—
HHPA	—	1 ml.	11 ml.
Cardolite NC 513	—	—	1 ml.
BDMA	1 per cent	1 per cent	1 per cent
Relative hardness		<	<

Hexahydrophthalic anhydride is solid at room temperature, and should be warmed to 55°C. before mixing with the resin brought to the same temperature. It is convenient to keep the infiltrating mixture in the graduated cylinder in which it is prepared to facilitate calculation of the amount of accelerator which is finally added. As the viscosities of the mixtures begin to increase after addition of the accelerator, the benzyldimethylamine should be added just before the final soaking prior to transferring the specimens to embedding capsules. The resin-accelerator-hardener mixture is best kept at room temperature until 15 minutes before transfer of specimens to capsules: at this point some advantage of reduced viscosity is gained by warming the mixture in the 40°C. oven.

Infiltrating Tissues with Epon 812

Fixation, dehydration, and infiltration of tissues are facilitated by gentle continuous agitation of small vials mounted on a disc rotated at 10 R.P.M. A typical preparative schedule is:

Fixation at room temperature	10 min.
Fixation at 8°C.	40 min.
Fixation at room temperature	10 min.
Wash in distilled water	2–3 min.
50 per cent alcohol	10 min.
70 per cent alcohol	10 min.
95 per cent alcohol	10 min.
Absolute alcohol	15 min.
(All subsequent treatments at 40°C.)	
Absolute alcohol/epon 812 (50/50)	15 min.
Epon 812	30 min.
Epon 812 plus hardener (2 changes)	1–2 hrs.
Epon 812 plus hardener and amine (2 changes)	1–2[8] hrs.

[8] If this time interval is appreciably extended, it is difficult to free the tissues and resin of trapped air bubbles after transfer to embedding capsules.

Water is appreciably miscible with epon 812 and the time of dehydration through the alcohols can consequently be abbreviated with safety. Since the viscosity of epon 812 is increased by addition of the hardener, the transition from absolute alcohol to the epoxy resin mixture is best accomplished by passing through the resin alone.

Sectioning Epoxy Resins

Tissues embedded in epon 812 can be trimmed and sectioned by techniques which have become routine for methacrylate embedded specimens. However, the harder epoxy mixtures are sufficiently rigid to resist plastic deformation in the microtome chuck, and there is consequently no need to cement specimens on bakelite or steel dowels.

Sections sufficiently thin to permit resolution of the primary and secondary filaments of striated muscle (Figs. 5 and 6) are readily cut with a sharp knife. Even such thin sections show only little or no compression, as judged by their retention of the block face dimensions and failure to spread when treated with solvent vapors (7, 8). If the knife is not of the sharpest, the elastic compression in sections may be relieved by xylene or chloroform vapors.

Two relatively minor sectioning difficulties have occurred sporadically. Sections at the knife edge are occasionally dragged back over the edge on the following cutting stroke, possibly as a result of accumulated debris at the edge or of a static electrical charge on the block. And at times, as sections are cut, they slide under the water surface: but since they usually return in a few seconds, no harm results if the water surface is kept clean.

Sections may be supported in the routine manner on films of formvar or parlodion (carbon-reinforced or not) mounted on grids. However, even the thinnest sections are exceptionally strong (if free from knife marks) under the electron beam and may be picked up on bare grids for examination without an evaporated layer of carbon to strengthen them (Fig. 4). Care must be exercised with unprotected sections, since abrupt exposure to high beam intensities will cause them to shrink and "clear" excessively.

The failure of protected epoxy sections to "clear" in the electron beam (Glauert) detracts somewhat from the *apparent* contrast on the microscope viewing screen, but this may be overcome by supplementary staining of sections with alcoholic (9) or aqueous (10) solutions of heavy metals. This

is not usually desirable, unless specific effects are required, for the appreciable increase in contrast of some structures leads to micrographic errors which completely lose structures of very low contrast. A more desireable approach is control of initial exposures so that plates can be developed for the manufacturer's full recommended time. The figures presented here (with exception of Figs. 5 and 6) are all of "unstained" sections, the original plates (Eastman Kodak, medium contrast) having been developed at 68° F. for 2.5 to 3 minutes in freshly prepared Eastman Kodak D-19.

Additional Comments

We have found the epoxy resins capable of uniformly preserving delicate structures (Figs. 2 and 3) which are often disrupted or otherwise damaged during embedding in the methacrylates.[9] This in itself recommends the epoxies as routine embedding materials. However, as with the methacrylates, chemically fixed specimens must still be subjected to the sometimes drastic shrinkage produced by alcohol dehydration. We are exploring the possibility of surmounting this difficulty by dehydrating tissues with a water-soluble component of epon 812, but our results are still too limited to be reported here.

References

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[9] Our attempts to embed osmium-fixed embryonic mouse and guinea pig livers in methacrylate have been consistently disappointing, in spite of the careful attention paid to many of the recommended variations (purification of monomers by fractional distillation, prepolymerization of monomer (11), incorporation of traces of uranyl nitrate (12), vacuum embedding (6), etc.). Pancreas, on the other hand, is well preserved by embedding in either type of resin.

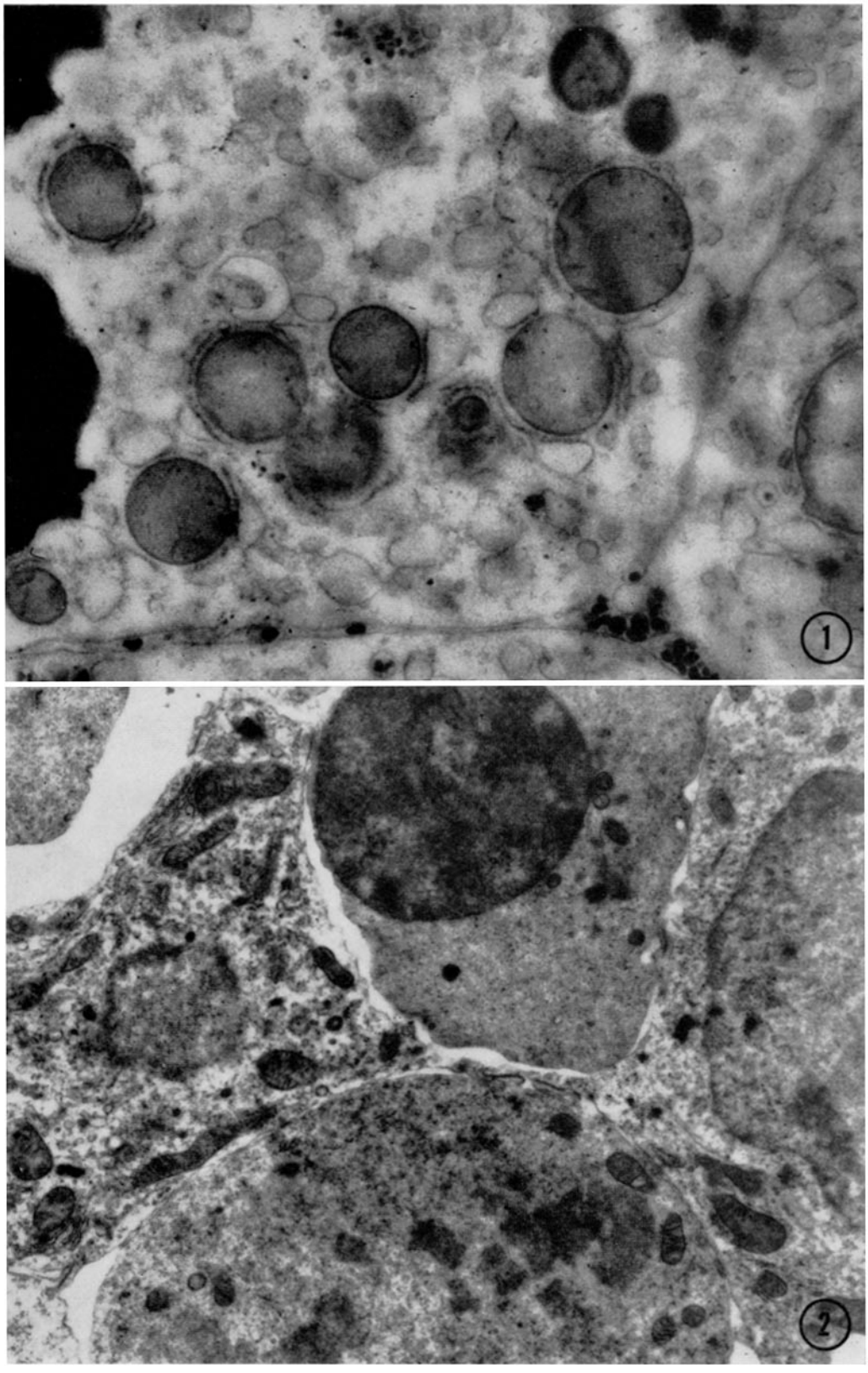
Explanation of Plates

Unless otherwise noted, all figures are electron micrographs of osmium-fixed tissues which have been embedded in epoxy resin mixtures based on epon 812; micrographs were taken of sections mounted on carbon-coated parlodion films and viewed without supplementary heavy metal staining.

Plate 5

Fig. 1. Section of embryonic guinea pig liver embedded in araldite 502. Portions of three apposed parenchymal cells are seen. Since the tissue was taken prior to the appearance of glycogen, no such deposits are present. Granular endoplasmic reticulum is scanty and restricted to crescent-shaped formations around the mitochondria. The agranular endoplasmic reticulum is prominent, as are fat droplets (right margin) and bile pigments (lower margin). Cell surfaces are closely applied to one another and do not show the shrinkage retraction commonly seen in methacrylate-embedded liver. ×17,000.

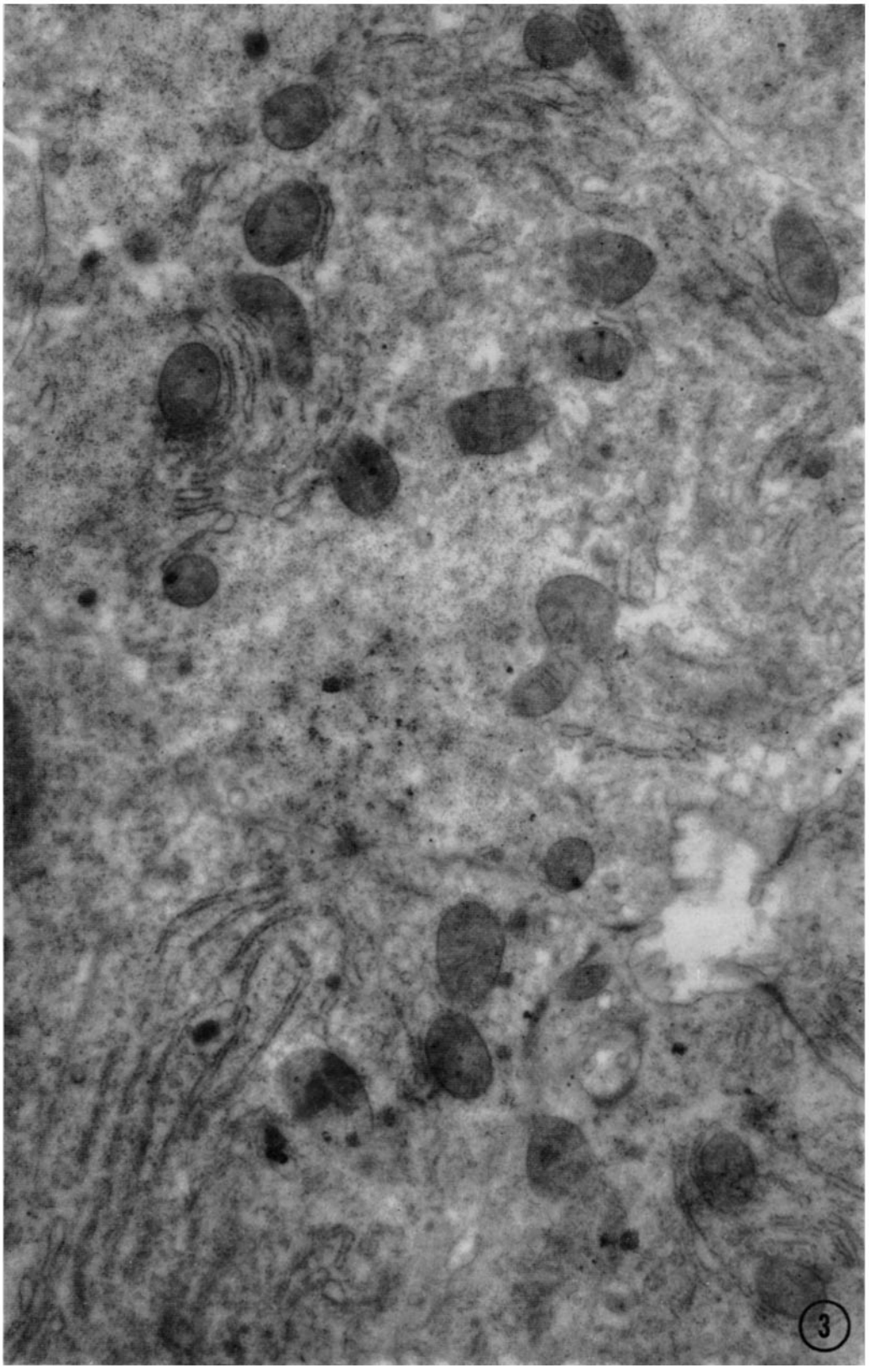
Fig. 2. Cluster of hemopoietic cells in a sinusoid of embryonic mouse liver. The dense nucleated cell in upper center is probably a normoblast. Cell outlines are well preserved and shrinkage-induced retraction is minimal. The nuclear contour is smooth and shows no deformation due to sectioning compression. × 8,000.



(Finck: Epoxy resins in electron microscopy)

PLATE 6

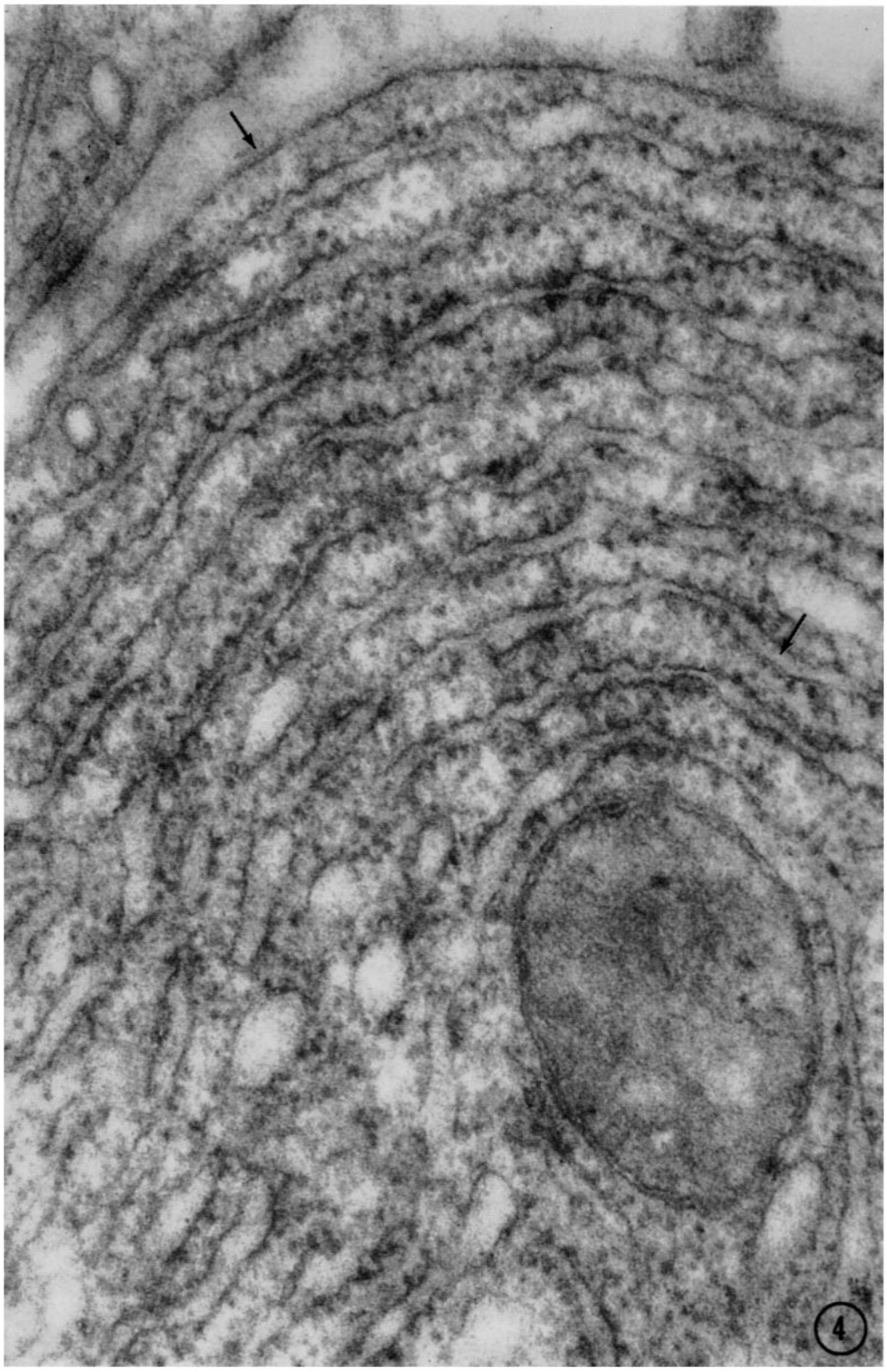
FIG. 3. Three embryonic mouse liver parenchymal cells clustered about a bile canaliculus. Cell outlines tend to be vague, but become more sharply defined as the canaliculus is approached and show the accumulation of dense material which typify the terminal bars (desmosomes) in this position. The delicate microvilli of the bile canaliculus are well preserved. Glycogen deposits, granular and agranular endoplasmic reticulum are well defined. × 10,000.



(Finck: Epoxy resins in electron microscopy)

PLATE 7

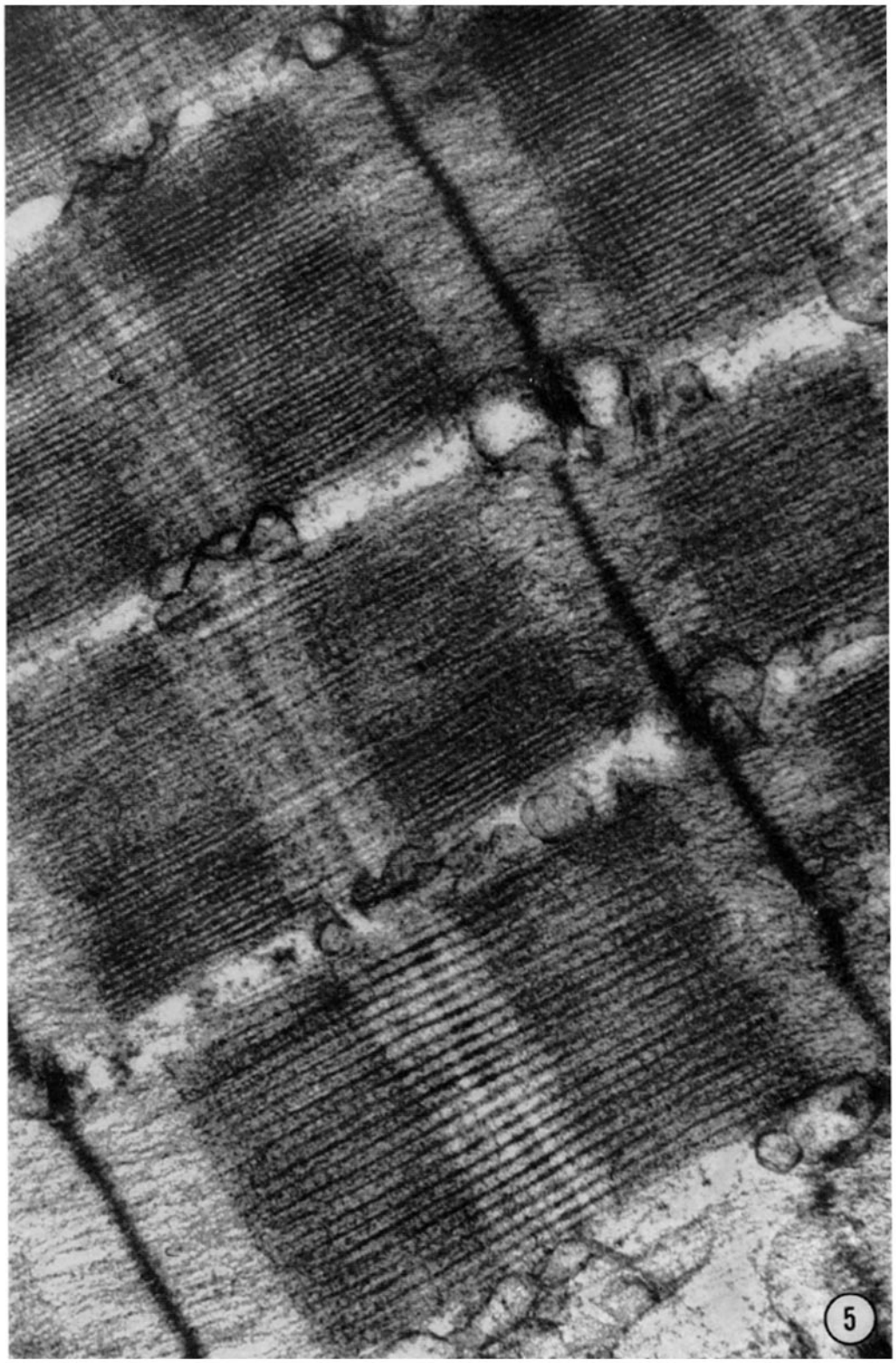
FIG. 4. Basal region of a mouse pancreatic acinar cell. Unprotected section mounted on a bare grid (without supporting film). A mitochondrion, cut transversely, occupies the lower right corner of the figure. Endoplasmic reticulum and attached nucleoprotein granules are quite prominent. Some areas (arrows) suggest that the membranes forming the cell surface and endoplasmic reticulum are doublets. × 170,000.



(Finck: Epoxy resins in electron microscopy)

PLATE 8

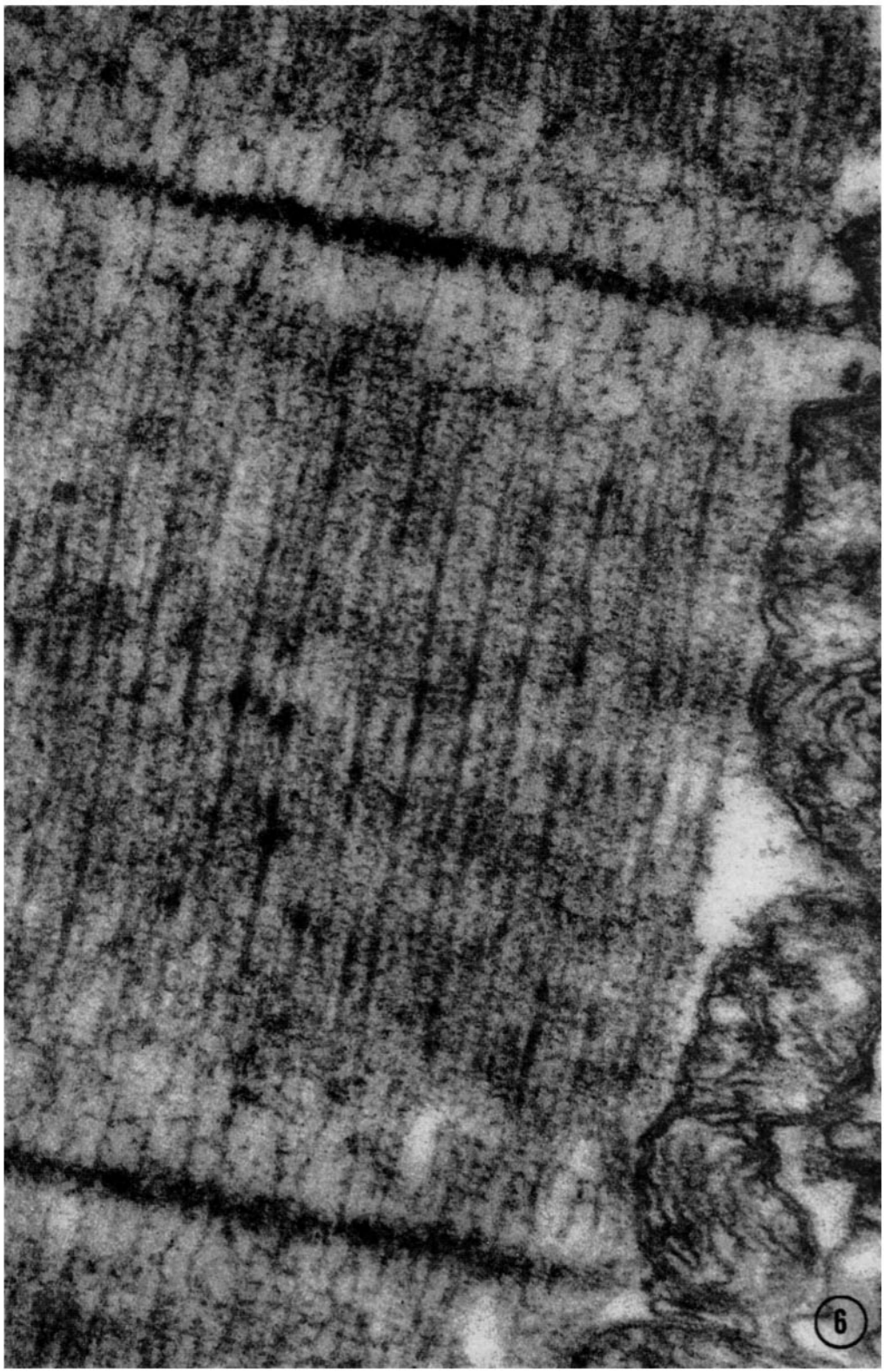
FIG. 5. Tail myotome of *Amblystoma* larva, embedded after osmium fixation; section stained for 8 minutes in 1 per cent phosphotungstic acid in 95 per cent alcohol. Orientation of the myofibril in the lower part of the figure is such that primary and secondary filaments (Huxley) can be seen. Note the difference in structure of the Z lines in the two upper myofibrils. In the uppermost fibril the secondary filaments appear to end at the apices of a continuous, zig-zag structure forming the Z line; in the lower fibril the Z line appears to be discontinuous, being represented by dense varicosities of the secondary filaments which seem to pass through uninterrupted from one sarcomere to the next. × 75,000.



(Finck: Epoxy resins in electron microscopy)

PLATE 9

FIG. 6. Mouse psoas major muscle. The osmium-fixed tissue was dehydrated for 10 minute periods in 50, 70, and 95 per cent alcohols, each containing 1 per cent uranyl nitrate. The section was stained for 3 minutes in 1 per cent phosphotungstic acid in 95 per cent alcohol. The relationship of primary to secondary filaments can be seen. × 135,000.



(Finck: Epoxy resins in electron microscopy)