

N-VINYLPYRROLIDONE AS A WATER COMPATIBLE CONSTITUENT OF EMBEDDING RESINS FOR SECTIONING IN ELECTRON MICROSCOPY

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ABSTRACT

The use as an embedding resin for ultrathin sectioning of a cross-linked triple copolymer of *N*-vinylpyrrolidone, acrylonitrile, and ethylene glycol dimethacrylate is described. The first of these components is miscible with water, in all proportions, and can be used as a dehydrating agent, or, alternatively, ethanol may be used in the standard way. Polymerization is carried out at 37°C or even lower temperatures. This resin is unsuitable for use after osmium fixatives, but after permanganates it gives results similar to epoxy. Photographs of rye root-tip cells fixed in permanganate and sectioned in this resin are presented. Because of the water-permeable nature of the product and low polymerization temperature, this resin appears to have possibilities for histochemistry.

Introduction

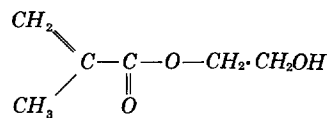
Normal epoxy and methacrylate polymers used in ultrathin sectioning are very water repellent. The liquid monomers (or partial polymers in the case of the epoxys) are also not water miscible, so that biological material has to be taken through water-ethanol or other dehydrating mixtures. For some histochemical purposes it may be desired to avoid this step, and a number of water compatible or partially water compatible substances have been proposed. Various difficulties are associated with their use. In all such cases, a monomer or prepolymer merely replaces ethanol or acetone or other dehydrating intermediate. Whether the solvent properties of the plastic monomer are preferable to those of the more usual dehydrating agents can only be decided on the basis of a particular histochemical problem. The same considerations apply to the plastic described in this paper, which

seems to have advantages over some of those proposed. It will be shown that with standard permanganate fixation it yields results similar to normal epoxy resins. The use for histochemical purposes has not been explored.

Review of Previous Work

Gibbons (1959) extracted the water miscible fraction of the epoxy Epon 812 (Shell); this fraction has received the name "aquon." It is very viscous, and only partially water miscible at temperatures above 15°C. Stäubli (1960) has reported trials with another water compatible epoxy, CIBA's "produit d'essai X 133/2097." The chemical nature of this product, and of the hardener and accelerator used with it, has not been divulged. It is also very viscous and penetration into biological materials is slow. Wichterle, Bartl, and Rosenberg (1960) and Rosenberg, Bartl, and Lesko (1960)

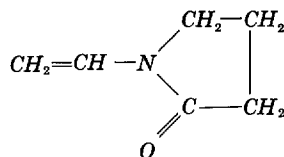
have used ethylene glycol monomethacrylate:



Unlike the two previous products, it is a true monomer and there are no penetration difficulties. In use, it is cross-linked with ethylene glycol dimethacrylate. The present writers have experienced difficulties with this monomer, apparently because it rapidly transesterifies to a mixture of ethylene glycol and its dimethacrylate. (Information kindly supplied by Dr. K. C. Tsou, of the Monomer Polymer Laboratories.) Scarpielli and Sittler (1959) have used polyvinyl alcohol. This is a fully polymerized, high molecular weight product at the time of embedding.

N-Vinylpyrrolidone and Its Copolymers

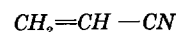
N-vinylpyrrolidone:



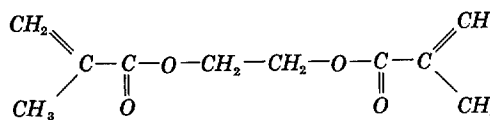
is a readily polymerizable monomer miscible with water in all proportions. It owes its water compatibility not to hydroxyls, but to the amide structure, which is also present in polypeptides. The polymer is a hygroscopic water soluble gum

which has been extensively used in blood plasma substitutes. Its remarkable tolerance by the body may be due in part to this polypeptide-like structure. It binds water, at least eight water molecules being bound by one amide group. Schildknecht (1952) may be consulted for a review of the extensive literature on *N*-vinylpyrrolidone and its polymers.

N-vinylpyrrolidone (hereafter abbreviated VPOD) is ordinarily polymerized by H₂O₂ in the presence of ammonia. The polymer is mechanically much too weak for use in sectioning. VPOD, however, readily copolymerizes with many acrylics. Numerous tests of such combinations were made. Particularly favorable properties are found in a triple copolymer consisting of VPOD with various proportions of acrylonitrile:



(hereafter called ACRN) and ethylene glycol dimethacrylate:



(hereafter called EGDM). A range of mechanical properties is obtainable by using different proportions. As a guide, one may say that increase of ACRN conveys toughness, while increase in EGDM, which is the cross-linking agent, produces hardness and brittleness. At least some EGDM is

Key to Labeling

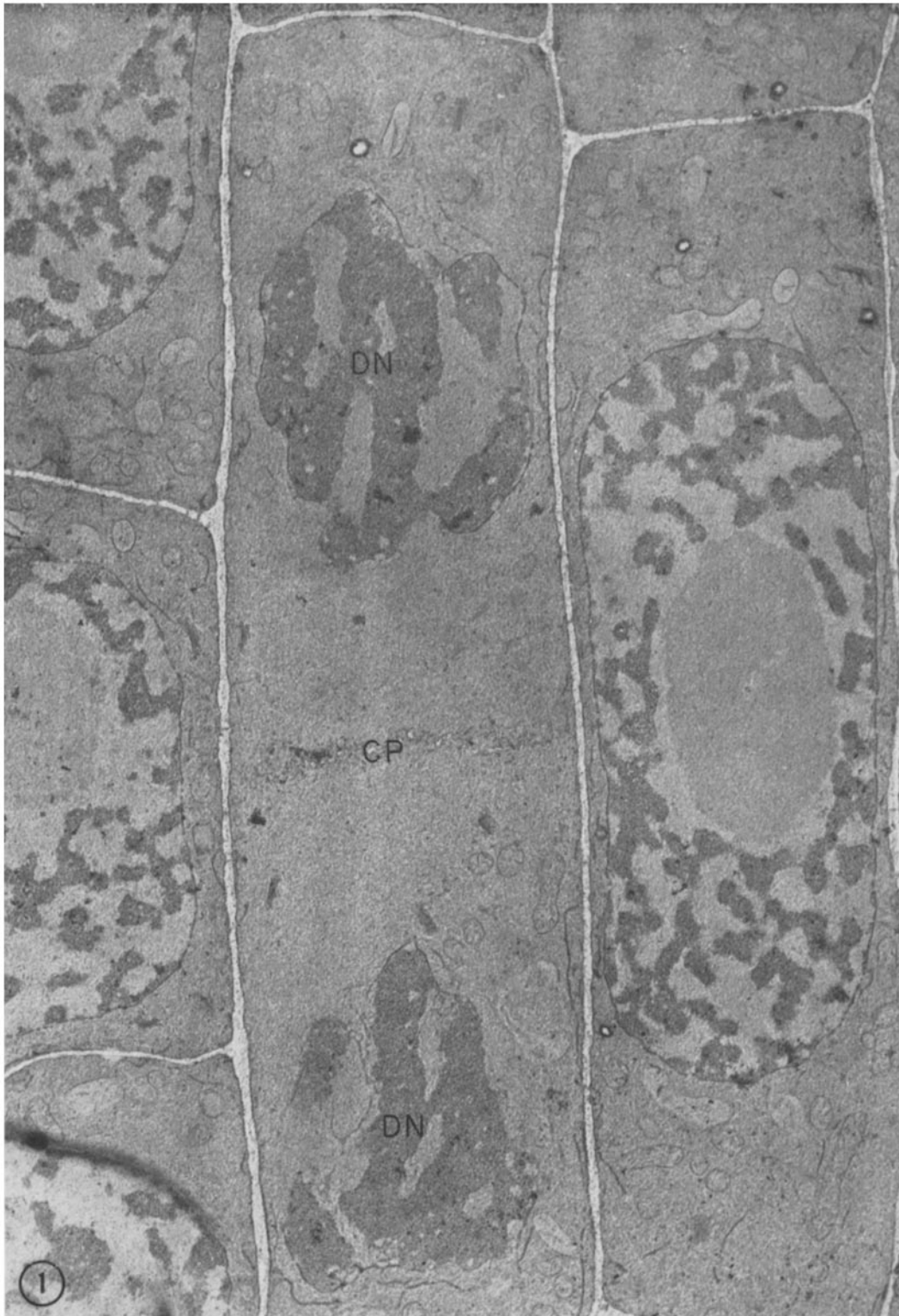
A, amyloplast
CH, chromatin
CP, cell plate
DN, daughter nucleus
ER, endoplasmic reticulum
GA, Golgi apparatus
GV, Golgi vesicle
II, unidentified inclusions

LV, lipid vacuole
M, mitochondrion
MVB, multivesicular body
N, nucleus
NE, nuclear envelope
NP, nuclear pore
P, phragmosome
W, wall

FIGURE 1

Section of rye root-tip fixed for 2 hours in 5 per cent aqueous unbuffered acrolein at 22°C, then rinsed in water, and postfixed for 2 hours in 2 per cent aqueous unbuffered KMnO₄ at 25°C.

This figure shows a dividing cell with two daughter nuclei (*DN*) and a new cell plate (*CP*). The deep chromatin stain seems to be characteristic for acrolein followed by permanganate. × 3800.



necessary to prevent melting of sections in the electron beam. More than 50 per cent by volume of ACRN may result in difficulties with separation of polymer. Poly-ACRN has the property of being totally insoluble in its own monomer, so that it cannot be homopolymerized in bulk; attempts to do this result in a precipitate. We have found the most desirable combination of properties in a copolymer of about $\frac{1}{3}$ of VPOD, $\frac{1}{3}$ ACRN, and $\frac{1}{3}$ EGDM by volume, but the proportions are not critical in this region and accurate measurement is unnecessary.

Specimen Preparation and Fixation Procedures

Several kinds of specimen were examined. Those illustrated are root-tips of fall rye (*Secale cereale* L.) variety Toivo. They were obtained by germinating the seed on moist filter paper in Petri dishes in the dark at room temperature and were harvested between 42 and 48 hours after sowing. Rye roots are very thin and for this reason it was not considered practical to split them for fixation. Instead the 2 millimeter tip portions were cut at about a 45° angle and these segments were processed.

Fixations used for the illustrations include the following. (a) 1½ hours in 2% aqueous unbuffered KMnO_4 at 10°C (Figs. 5 and 6). (b) 2 hours in 5% aqueous unbuffered acrolein, water rinse, then 2 hours in 2% aqueous unbuffered KMnO_4 , all at 25°C (Figs. 1, 2, and 7). (c) 2 hours in 3 per cent aqueous unbuffered formaldehyde, water rinse, 1½ hours 2 per cent aqueous unbuffered KMnO_4 , all at 25°C (Figs. 3 and 4). After the above procedures, the material was washed in water and dehydrated through the standard water-ethanol

mixtures. From ethanol it was transferred to the mixture of the three monomers.

Embedding Procedure

The mixture used was $\frac{1}{3}$ VPOD, $\frac{1}{3}$ ACRN, $\frac{1}{3}$ EGDM by volume; after two changes of 5 to 10 minutes each the material was placed in the final mixture containing 1 per cent benzoyl peroxide. This mixture polymerizes to a hard solid, with 1 per cent benzoyl peroxide as catalyst, in about 3 days at 37°C. Polymerization is possible at 25°C, in about twice the time. Removal of oxygen is not necessary. After polymerization at 37°C the blocks were removed from their tubes and left for a further 24 hours at 50–60°C to remove possible volatile constituents, but we are not certain that this ripening step accomplishes a useful purpose. Because of the volatility of ACRN, polymerizing tubes have to be well corked and gelatine capsules are inconvenient. We have used small serological glass test tubes, 50 mm long, 6 mm OD (EXAX culture tubes without lip 45060, Owens-Illinois). Material was allowed to sink to the bottom of these tubes, but if for some reason this is not wanted, a disc of thick filter paper (*e.g.* No. 17 Whatman), accurately cut out with a punch, can be pushed into the tube to make a false bottom.

This highly cross-linked copolymer is always formed from the bottom of the tube upwards, passing from liquid to solid through a definite boundary, without any intervening viscous stage. When the polymerization is half-completed, the bottom part of the tube will be solid, the top liquid. A good heat sink is essential for regular polymerization, to conduct away the heat of the very exothermic reaction (about 22 kilocalories per mole). We have accomplished this as follows. Up to eight culture tubes (each one corked) are

FIGURE 2

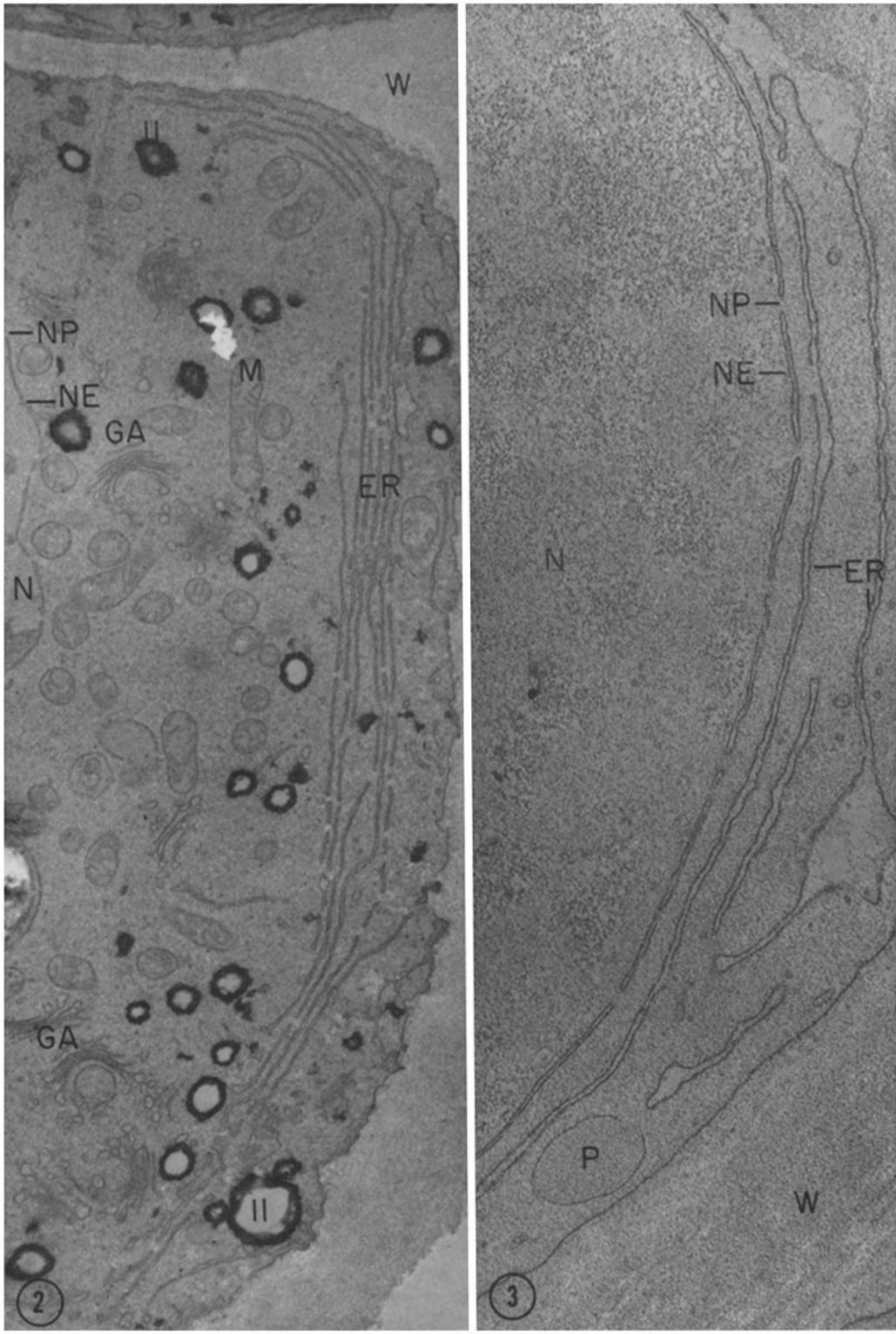
Section of rye root-tip fixed as in Fig. 1.

This figure demonstrates the appearance of nucleus (*N*), nuclear pores (*NP*), Golgi apparatus (*GA*), mitochondria (*M*), the profiles of endoplasmic reticulum (*ER*), unidentified inclusions (*II*), and wall structure (*W*). $\times 13,000$.

FIGURE 3

Section of rye root-tip fixed for 2 hours in 3 per cent aqueous unbuffered formaldehyde at 22°C, then rinsed in water and postfixed for 1½ hours in 2 per cent aqueous unbuffered KMnO_4 at 25°C.

This figure shows a nucleus with a characteristic pattern of variable granular substances (*N*), nuclear envelope (*NE*), nuclear pores (*NP*), endoplasmic reticulum (*ER*), with vacuolar areas, a phragmosome (*P*), and wall structure (*W*). $\times 20,000$.



stood inside a shell vial, 1 inch in diameter, $3\frac{1}{2}$ inches long. On the bottom of this shell vial is a layer of 3 or 4 mm of some non-volatile inert liquid to ensure good thermal contact. Water is unsuitable, but triethylene glycol or glycerol serves well. The shell vial is also corked and is stood in an incubator at 37°C.

This polymer does not stick to glass, but the particular culture tubes used had a slight inward lip, which made it necessary to break them. The specimen must be cut out of the block with a jeweler's saw, with the block held in a small vise. It is glued to a lucite rod, $\frac{5}{16}$ inch in diameter, with Eastman 910 adhesive. Most of the trimming is done with a grade 6 Grobet triangular file.

Mechanical Properties for Sectioning

When many polymers, covering a range of properties, are tested for cutting properties in the ultramicrotome, an unexpected relationship appears. Other things being equal, materials that are most brittle macroscopically cut best. The same relationship has been observed with a range of about 30 waxes (Fabergé, 1949). We suspect there may be a fundamental underlying reason, connected with the dimensional scaling of the process of sectioning. With reference to section thickness, the factor is about 100; unfortunately, the mechanics of microtome sectioning cannot in the present state of knowledge be reduced to fundamental physical parameters. It may be noted that even with a much smaller change in scale, large steel specimens are more prone to notch brittleness than small ones, a subject that has received extensive theoretical treatment. A discussion of these problems will be found in Orowan (1949). The practical rule is that a plastic should not be passed over as a possible sectioning resin because it appears too brittle. An actual test in the ultramicrotome should be made.

Wetting of Knife

A diamond knife was found more satisfactory to cut this resin. In common with all other water

compatible plastics tested, it will wet the back of the knife when the usual collecting trough with water is used. This is easily overcome by painting the four sides of the block, after it has been finally trimmed and mounted in the microtome, with a solution (about five per cent) of ordinary histological paraffin in any volatile solvent, such as benzene. A few moments must be allowed for the solvent to evaporate. This measure is equally effective against wetting for ordinary embedding media.

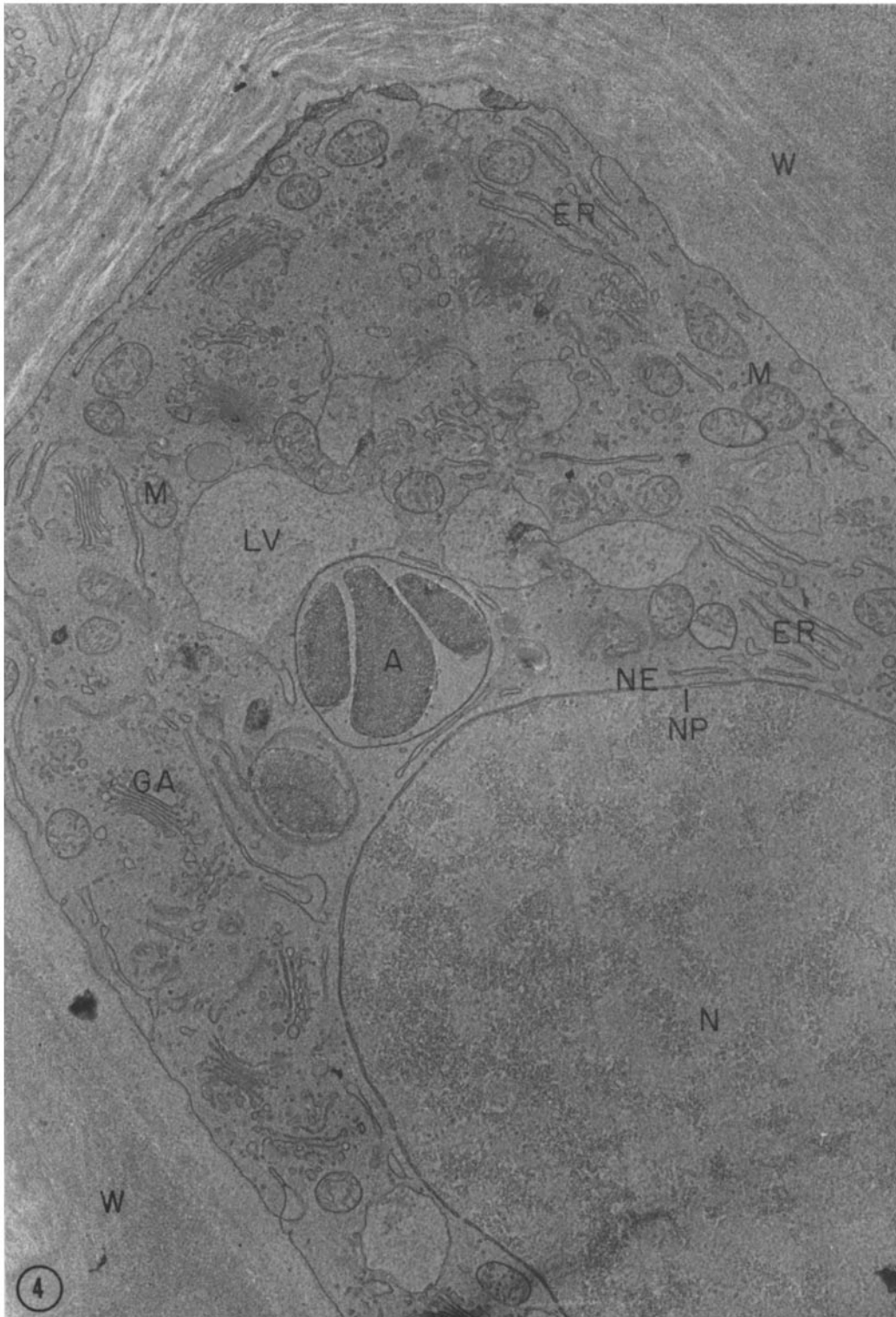
Other Properties

Thin silver-grey sections are obtainable. They will float on water and can be collected in the usual way, but the forces of spreading are much less strong than with water repellent media. In attempts to improve spreading, the addition of several possible plasticizers was tried, including dibutyl phthalate and cetyl alcohol; these did not produce any worth-while improvement, but the addition of 1 or 2 per cent of Formvar 7/70 (Shawinigan Resins Corp., Springfield, Massachusetts) made the sections much better. Formvar 7/70 dissolves in the VPOD-ACRN-EGDM mixture in a few minutes, and was only added in the last embedding stage. Preparations of bacteria and unicellular algae in the VPOD-ACRN-EGDM copolymer showed good structural detail, but had a gap on part of the periphery between the outer wall and the surrounding plastic. Varying the proportions of the constituents does not change this. Whether this is due to actual shrinkage during polymerization or to absorption of water during cutting has not been determined. Sections of root (as demonstrated) and animal tissue showed no artifacts attributable to shrinkage. So called "explosion" artifact has never been seen. The resin does not melt in the beam, behaving in this respect like epoxy. We believe that melting in the beam will not occur with cross-linked polymers generally since these have no melting points and remain rigid until chemical breakdown at a high temperature. Sections can be therefore either supported

FIGURE 4

Section of rye root-tip fixed as in Fig. 3.

This figure shows a nuclear structure like that in Fig. 3, as well as other inclusions: Golgi apparatus (*GA*), mitochondria (*M*), the profiles of endoplasmic reticulum (*ER*), amyloplast (*A*), and lipid vacuoles (*LV*). Wall structure (*W*) is seen surrounding the cell.



on the usual carbon-coated collodion, or else used unsupported.

Poststaining of sections has been tried, using potassium permanganate and other permanganates, uranyl nitrate, and lead acetate. In all cases, reduction rather than increase in contrast resulted. Presumably this relatively water permeable medium absorbs all of these staining materials. VPOD specifically binds iodine (attributed to Shelanski) and treatment of mounted sections in I and KI results in a strong negative stain. These observations lead to the conclusion that physical absorption of electron stains may at times be more important than any true chemical specificity. There is no doubt that poststaining sections in epoxy, which is extremely water repellent, is much easier than poststaining the plastic here described. One might suggest that in epoxy, structures may act as wicks to absorb electron stains much more readily than the surrounding plastic, for purely physical reasons and not because of chemical specificity.

Osmium-fixed preparations, handled in the standard manner, cannot be embedded in VPOD containing plastics, apparently because of a reaction between this and osmium compounds leading to loss of contrast. Permanganates, on the other hand, can be used in a variety of ways.

Acrolein followed by permanganate seems to darken chromatin quite deeply.

Preparations have been made either by dehydrating through water-ethanol or through water-VPOD mixtures. A careful comparison of many photographs shows no consistent difference between the two dehydrating methods. The material used for the illustrations was processed through ethanol.

All three components, VPOD, ACRN, and EGDM, are kept without inhibitors and used as received; they should be kept in a refrigerator. The mixture is made up only before use. Persons handling ACRN should be aware that it is very toxic, but a hood or other special precautions are not necessary on this scale. VPOD and EGDM are obtainable from the Monomer Polymer Laboratories, the Borden Chemical Company, Philadelphia, Pennsylvania.

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FIGURE 5

Section of rye root-tip fixed for 1½ hours in 2 per cent aqueous unbuffered KMnO_4 at 10°C.

The figure shows Golgi apparatus (*GA*), mitochondria (*M*), profiles of endoplasmic reticulum (*ER*), an amyloplast (*A*), phragmosomes (*P*), lipid vacuoles (*LV*), and wall structure (*W*). $\times 18,000$.

FIGURE 6

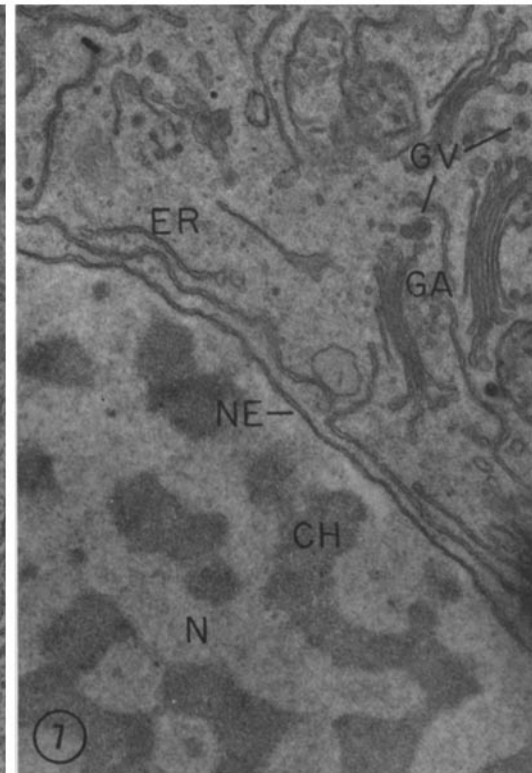
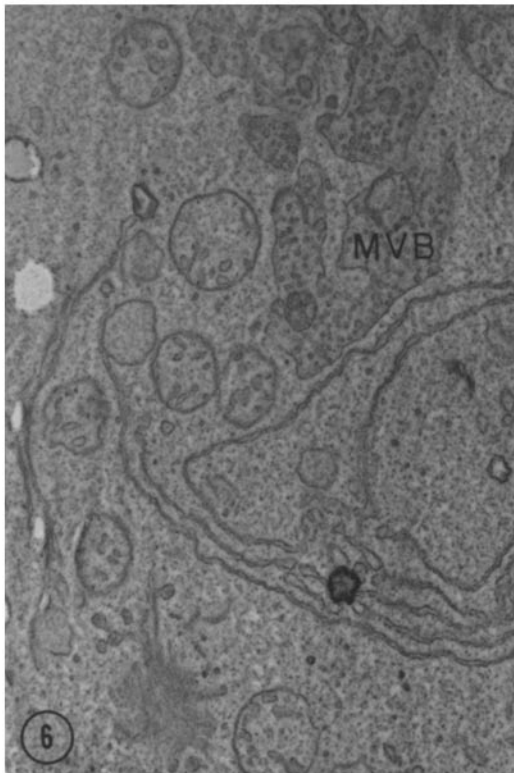
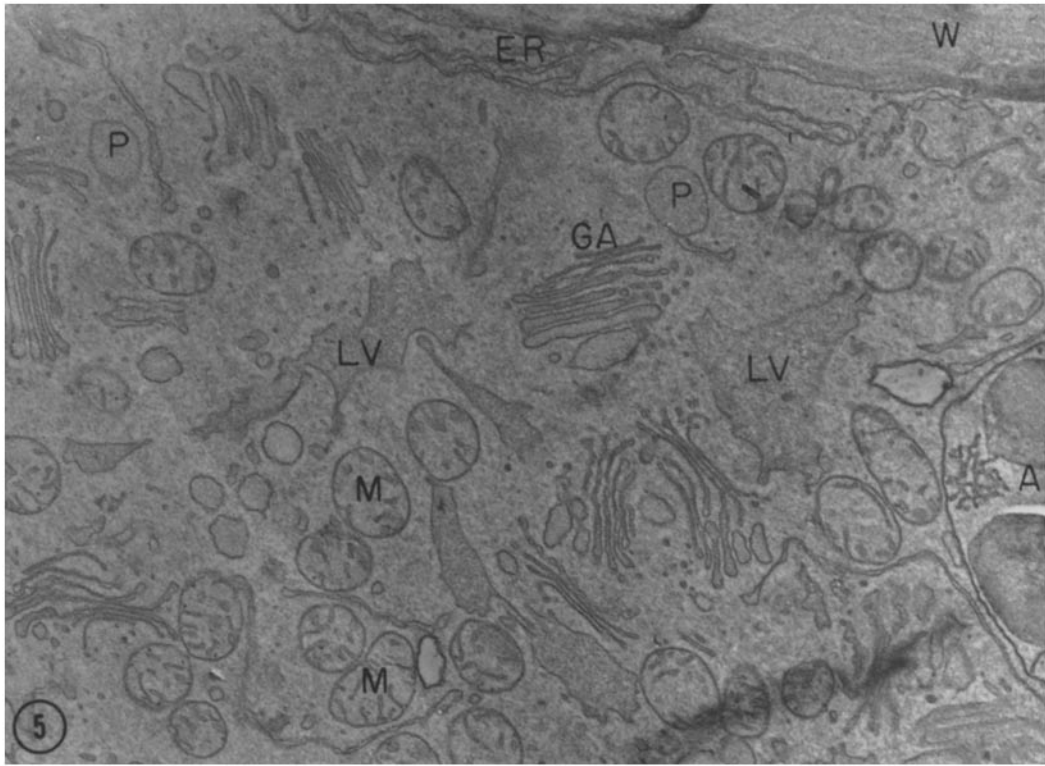
Section of rye root-tip fixed as in Fig. 4.

The figure shows, in particular, multivesicular bodies (*MVB*). $\times 18,000$.

FIGURE 7

Section of rye root-tip fixed as in Fig. 1.

The picture shows a nucleus (*N*) with organized areas of chromatin (*CH*), the nuclear envelope (*NE*), profiles of endoplasmic reticulum (*ER*), and Golgi apparatus (*GA*) with associated Golgi vesicles (*GV*). $\times 18,000$.



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