guished from normal and from the homozygote, it is possible to predict with a high degree of accuracy which marriages should result in homozygous individuals—in this case, children with sickle cell anemia. Since (homozygous) individuals with sickle cell anemia either die young or, if they reach maturity, have a greatly lowered fertility, the vast majority of cases of the disease are the issue of marriages between two (heterozygous) persons with the sickle cell trait. In the absence of marriage between individuals whose erythrocytes exhibit the sickling phenomenon, the frequency of the homozygote would greatly decrease, and sickle cell anemia would tend to disappear, with only a very rare case arising as a result of mutation in a normal individual married to a person homozygous or heterozygous for the sickling gene.

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TECHNICAL PAPERS

New Sectioning Techniques for Light and Electron Microscopy

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The application of the electron microscope to many biological problems has been seriously hampered by the lack of a rapid practical method of cutting uniformly thin sections having adequate area and integrity of structure. Because of the very slight penetrating power of the beam in commercial electron microscopes and the great relative depth of field involved, specimen structure is difficult to interpret when sections are over a fraction of a micron in thickness.

Although various solutions to this problem have been described in the literature, their success and general application have been limited. A departure from classical approaches to the problem has been the high speed microtome (*2*, *3*). However, this precision equipment is not only expensive and complicated but produces a low percentage of usable sections. Moreover, the sections are cut so rapidly and abundantly that selection is time-consuming and uncertain. Several workers (*1*, *5*) have described a technique which uses the thinnest portions of wedge-shaped sections for electron microscopy. Their methods, however, have been laborious and difficult to reproduce.

The most recent effort has been that of Pease and Baker (*4*), who have used standard histological techniques to embed tissue in collodion and paraffin. For sectioning, they altered a Spencer rotary microtome so that the unit of advance was reduced to approximately one-tenth the calibrated value. The microtome was then reported to produce sections as thin as 0.1 μ. Many workers, however, have had trouble in using their technique, mainly because of the exacting demands made on the microtome-advancing mechanism. Another disadvantage has been the difficulty of making very thin sections with the standard embedding media, such as paraffin and collodion.

In recognition of this problem, a promising new development in ultramicrotomy is presented. It consists of a method for obtaining extremely thin sections, involving the use of a methacrylic resin as an embedding medium, a thermal expansion device for advancing the specimen in a commercial microtome, and metallic shadow-casting for increasing observable detail in some of the sections. These techniques form the basis of a new method for producing numerous thin sections suitable for obtaining transmission images at the higher magnifications in the conventional light, phase-contrast, and electron microscopes. Polymerization of *n*-butyl methacrylate provides a rapid and simple means for embedding the fixed biological material in a solid resin. This gives an optically clear matrix from which the sections are cut, one at a time. Smooth, continuous advance of the embedded

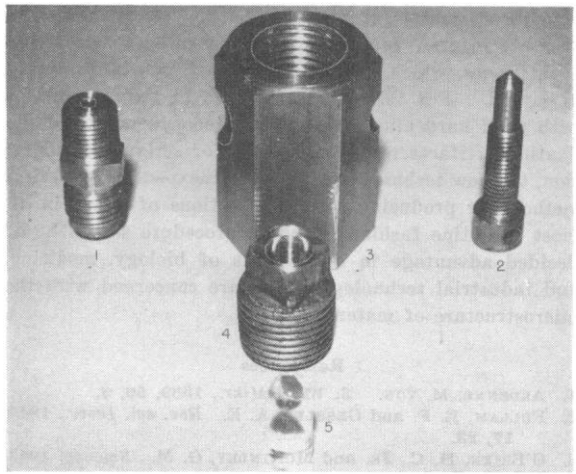

FIG. 1. Disassembled thermal expansion apparatus: (1) and (2) needle valve for carbon dioxide gas, (3) brass expansion chamber tapped for the fittings, (4) standard 3/8-in. brass pipe plug with cavity in face to seat embedded tissue, and (5) tissue in the clear resin with gelatin capsule removed.

specimen toward the knife is effected by the thermal expansion of a brass specimen holder, which permits the Spencer microtome, with its advancing mechanism disengaged, to cut ultrathin sections having uniform thickness, large area, and integrity of tissue structure.

Before being embedded in the polymer, the tissues are fixed and then dehydrated in an ethyl alcohol series by the usual cytological techniques. From absolute alcohol they are transferred to a solution containing equal volumes of absolute alcohol and pure monomeric *n*-butyl methacrylate from which the inhibitor has been removed. After about 1 hr in the alcohol-monomer mixture, the tissues are put in the monomer alone for an equal period. To ensure removal of the alcohol, they are then placed in two additional changes of monomer for at least 1 hr in each.

No. 00 gelatin capsules are convenient embedding molds. The main body of the capsule is set upright in a wooden block or other base and filled with the monomer, to which has been added 1% (by weight) of a catalyst (2,4-dichlorobenzoyl peroxide). After the tissue is placed in the mixture, the capsule lid is slipped on to retard evaporation, and the assembled capsule is placed in an oven kept at a temperature of 45° to 50° C. For even heating, the capsules are suspended by strips of cellophane tape and good air circulation is maintained in the oven.

At the end of 6–8 hr the monomer is polymerized into a solid matrix containing the tissue embedded at the bottom of the clear plastic. An additional period of several hours at this temperature will ensure complete cure. After soaking in water, the gelatin capsule may be peeled from the resin.

An inexpensive device is used for holding and advancing the embedded specimen (Fig. 1). It is essentially a brass block containing a hole threaded at one end to receive a standard ⅜-in. brass pipe plug. A cavity drilled into the face of the plug provides a seat for the embedded specimen. Behind the plug is a needle valve, which admits compressed carbon dioxide. As the gas undergoes a large change in volume, it cools and contracts the assembly. Stopping or reducing the flow of gas allows the apparatus to approach room temperature again and thus provides continuous advance of the embedded tissue toward the cutting edge.

In practice, the embedded specimen is first cemented into the mounting block with a mixture of pure gum rubber and paraffin. Then, with the device clamped in the jaws of the microtome head, the entire assembly is cooled below room temperature. Upon the appearance of a thin layer of frost on the metal, the knife is adjusted so that the specimen just misses it on the cutting stroke. The specimen is then mechanically advanced at 2- or 3-μ increments until the first slice is made. At this point the mechanical advancing mechanism of the Spencer rotary microtome is disengaged by setting it to zero and the gas flow is reduced or stopped. After a few seconds the specimen can be cut again. Because the specimen is advancing continuously, a quick chopping stroke involving one complete revolution of the hand wheel is necessary. With a little experience one can soon judge the necessary time interval between cuts. Some control of the rate of specimen advance can be obtained by bleeding the carbon dioxide at various reduced rates into the expansion chamber.

Although polybutyl methacrylate has excellent cutting properties, the sections usually are found to be somewhat folded. They are lifted from the knife with a dry camel's hair brush, picked up with a dissecting needle, and placed on a water surface warmed on a hot plate to about 35° C. After a period ranging from a few minutes to an hour or more, many will flatten out on the surface and exhibit bright interference colors. These sec-

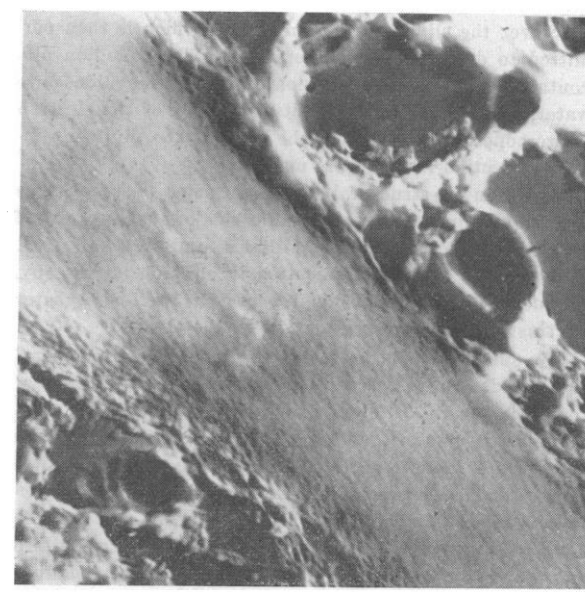

FIG. 2. Frog's eye, section through cartilage, fixed in Bouin's. Electron micrograph, total magnification × 10,400.

tions are then floated onto clean microscope slides and allowed to dry flat. Sections prepared for phase contrast microscopy are placed in acetone or toluene for about ½ hr to remove the matrix and are mounted in Canada balsam. For ordinary light microscopy the matrix is dissolved off and the section stained in the usual way.

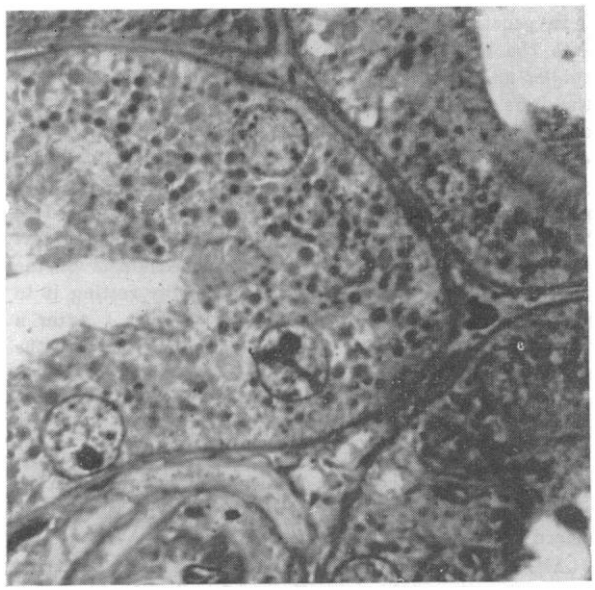

FIG. 3. Dog kidney, tubules and glomerulus, fixed in Zenker-formol. Electron micrograph, total magnification × 1500.

In preparing material for the electron microscope, the sections are floated from the water onto clean glass slides and dried flat. The matrix is then dissolved out by placing the slide in acetone, toluene, or amyl acetate. A dilute solution of collodion in amyl acetate is allowed to flow over the slide bearing the tissue, which is then permitted to dry at room temperature. The collodion film containing the section is floated from the slide onto water, and the specimen-mounting screens of the electron microscope are placed over the area of the film containing the section in the usual manner.

Detection of structural details in some of the tissues was greatly improved by metallic shadow casting of the sections as described by Williams and Wyckoff (*6*). This process, of wide application in electron microscopy, produces a three-dimensional aspect as well as greater contrast in the structural details of the tissue.

The suitability of the sectioning technique for general use was determined by preparing a variety of biological tissues in several different fixatives (Figs. 2 and 3). Although this new method for obtaining very thin sections has given satisfactory results, it possesses certain limitations. Knife sharpness, for example, is of vital importance. However, the tilt of the knife during sectioning is not particularly critical. The greatest chance for failure appears to lie in the polymerization of the embedding mass. Use of low temperature catalysts and maintenance of a curing temperature of 45° to 50° C will usually prevent the formation of insoluble resins. Occasionally tissues are injured during the polymerization reaction and such tissues, which are easily detected, can be promptly discarded. For this reason, the use of fixing solutions with good hardening properties is recommended. While fixation artifacts remain problems for serious consideration, the new technique provides an inexpensive, practical method for producing ultrathin sections of tissue in almost a routine fashion. Such a procedure should be of decided advantage in those fields of biology, medicine, and industrial technology which are concerned with the microstructure of materials.

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Protection of Lupulon and Humulon by Ascorbic Acid

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Lupulon and humulon, two antibiotics of known structure (*3*, *4*) obtainable from hops (*6*), are of special interest because of their activity against *Mycobacterium tuberculosis* (*1*). Both are active *in vitro;* lupulon is active *in vivo* as well. Both show activity against several acid-fast organisms (*1*), against other Gram-positive bacteria (*4*), and against fungi (*2*). They lose activity, in part by oxidation, when boiled in aqueous solutions (*5*).

The possibility of increasing the stability of these substances was investigated for several reasons. It is desirable to have a method by which they can be heat-sterilized in water solution. Also, it has been noted that their antibiotic activity in nutrient media is often temporary, perhaps because of instability at incubation temperature. In brewing, humulon suppresses the growth of certain undesirable bacteria (*6*). However, nearly all of the lupulon and much of the humulon is inactivated during boiling of the beer wort (*7*). If these antibiotics could be adequately stabilized, their effectiveness would increase and their use might be extended to foods.

Humulon and lupulon were prepared as previously described (*2*). For preparation of aqueous solutions, 40 mg of the antibiotic was first dissolved in 1 ml of warm propylene glycol. Phosphate buffer (0.015 M, pH 6.5)

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