Sectioning of Tissue for Electron Microscopy[1, 2]

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PEASE AND BAKER (*3*) made a major contribution to the use of the electron microscope when they showed that it is possible to cut sufficiently thin sections of tissue by relatively simple modifications of conventional microtomes and techniques. The early results obtained by these workers and by others who have followed their technique, or modifications of it, have shown, however, large-scale and obvious artifacts that detract considerably from their value to the cytologists and pathologists who might ultimately be interested. Since these artifacts were somewhat greater than are normally encountered in conventional light microscopic techniques, it could be anticipated that they arose either in the ultra-thin sectioning or in the subsequent handling of the specimens.

Improvements in Ultra-thin Sectioning of Tissue

In the present work an analysis was made of these aspects of the Pease and Baker technique in order to identify and eliminate the sources of the artifacts. The results of the analysis are presented briefly in the following pages, but a more detailed report will be presented elsewhere (*2*).

Since the aim of the present research was to study the cutting and mounting of the sections, an attempt was made to eliminate the variables involved in the selection, fixation, and embedding of the tissue by studying a single set of blocks originating from one piece of tissue. As the best micrographs in the literature appeared to be those in Pease and Baker's original work, the same type of specimen and identical embedding techniques were adopted. The specimen[3] consisted of a normal mouse liver perfused for 24 hours with 1 percent osmium tetroxide in physiological saline. It was then cut into pieces a few millimeters on the side. These were dehydrated through alcohol, embedded in 12 percent collodion, hardened with chloroform, cut into 1-mm cubes, mounted and impregnated with 60° paraffin. Since all the steps were carried out simultaneously, the only likely variation would result from the low penetrating power of the osmium. The results indicated that the fixation was quite uniform. Having uniform blocks eliminates a large number of variables and greatly simplifies the sectioning problem, which can then be divided into three distinctly separate steps to be considered independently. These are (1) the advancing of the block by uniform and specified amounts, (2) the cutting of the sections, and (3) the mounting of the sections.

1. *Block advance.* In the Spencer rotary microtome, unmodified except for the changed angle of the inclined plane, the variations in block advance had a mean value between 0.1 μ and 0.2 μ. These variations were found to be due to external vibrations, irregularities introduced by manual operation, static friction in the screw and associated parts, and static friction in the horizontal slides. To eliminate these effects, the following changes were made: (*a*) A motor drive with a vibration-free coupling was added. (*b*) The unit advance was set at 0.02 μ, so that the motions in the screw, screw bearings, and pointer bearings were all large for normal section thicknesses of 0.2 μ. This also reduced the effect of inaccuracies in the screw. (*c*) Ninety percent of the weight of the inclined plane and the attached block support was removed from the horizontal slides by means of a leaf spring. In the microtome used, there is a horizontal excursion of the block amounting to 300 μ in each cycle, which removes most of the static friction from the horizontal slide system. The last modification reduces the static friction that enters when the horizontal movement of the block stops at each end of the cutting cycle. As a result of these modifications, the variations in the magnitude of the advance have been reduced to less than 0.01 μ.

Thermal drifting of the block introduces some error of calibration but does not produce variations in thick-

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[2] Based on a paper presented at the Washington, D. C., meeting of the Electron Microscope Society of America, October, 1949 (Abstracts 13 and 14, *J. App. Phys.*, 1950, **21**, 67).

[3] The authors wish to thank J. J. Biesele and J. A. Jacquez for providing carefully prepared specimens and for their many helpful discussions.

ness when the microtome is motor-driven. The change in calibration was found to be negligible if lights and drafts were kept from the instrument. To check for thermal drifting, it has been found most convenient to retract the block a known number of cycles while the sections are being cut, and to observe whether the first new cut is made on the correct cycle. By noting the error and its polarity, it is a simple matter to compute the true amount of the advance. It is now considered that the thickness of the layer removed from the block is known to within 10 percent.

2. *Cutting.* Very little reliable information has yet been obtained with regard to the optimum values of the various parameters that concern the actual cutting of the block. In the work presented here, commercially sharpened knives were used and proved reasonably satisfactory for 0.2 μ sections, though a smaller number of localized defects in the edges would be desirable. Contrary to the experience of Pease and Baker (*1*), the cutting was found to be very insensitive to the angular setting of the knife. This may be due to the use of the liquid-reservoir method of collecting specimens. The remaining aspects of the cutting that were studied are described below and in the more detailed papers (*3*).

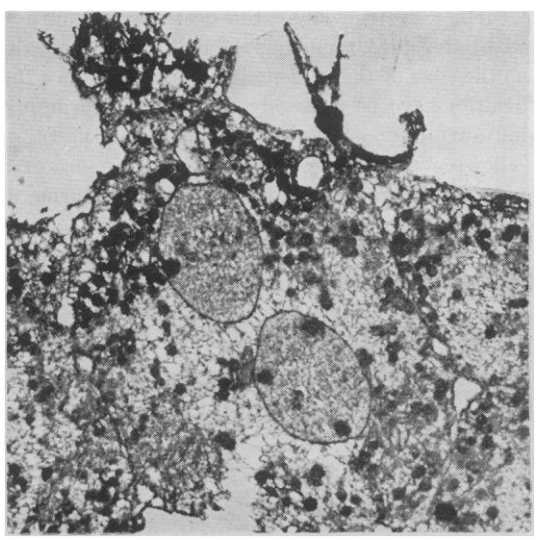

FIG. 1. Normal mouse liver fixed by perfusion with 1% osmium tetroxide in physiological saline. Section thickness 0.2 μ. Embedding was completely removed and replaced by thin collodion membrane for support. Note general distortion of cytoplasmic components leaving open network structure. Approximately × 2500.

3. *Mounting sections.* In the early work, the most generally used method of mounting sections after cutting was to remove the embedding materials with appropriate solvents and to replace them with a very dilute collodion solution that acted as a support for the dried tissue sections. A study of a few serial

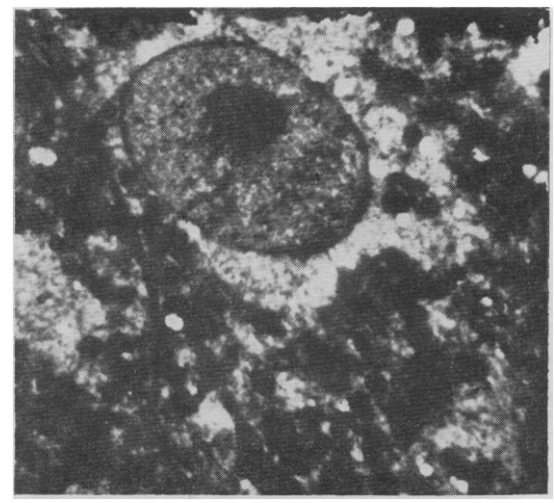

FIG. 2. Same specimen and section thickness. In this case embedding was completely removed and replaced with sufficient collodion to provide film slightly thicker than section. The surface tension effects producing the distortions in Fig. 1 are now absent. However, comparison with Fig. 3, *B* shows that finer particles have migrated and attached themselves to the coarser structures. Note increased thickness of the cell and nuclear membranes, and general matting of the cytoplasmic structures. Approximately × 5000.

sections quickly revealed that the surface forces acting on the tissue in this method produced a typical distortion that was very often large-scale and was primarily responsible for the defects observed in the early work (Fig. 1). This was modified by replacing the embedding material with an amount of collodion that would dry to a film somewhat thicker than the section. This eliminated the distortions caused by surface forces, but greatly reduced the contrast obtainable in the electron micrographs. Contrast was improved by the use of longer focal length objectives with small limiting apertures (50 μ diameter). However, successive serial sections still revealed changes that appeared to be due to a migration of the unsupported smaller structures during the dissolving of the embedding medium (*cf.* Figs. 2 and 3*B*). This discovery led to the simplest and most effective of all the techniques, namely, giving the sections no treatment whatever.

The present technique consists of mounting a 200A clear collodion membrane on a square inch or more of 200-mesh copper screen (etched to give more than 50 percent open area) and, after drying, using appropriately sized pieces of it to lift the sections directly from the liquid in the reservoir. The extra collodion membrane serves to anchor the sections to the screen.

With these techniques a large number of very consistent micrographs of the mouse liver have been obtained. Fig. 3 is a typical example. Some artifacts are still introduced, consisting mainly of "knife

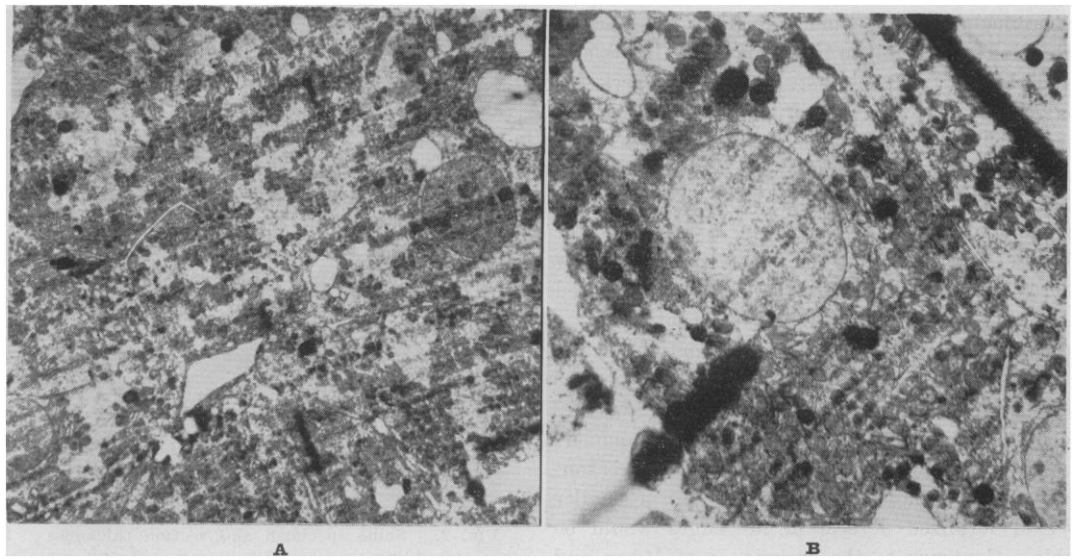

FIG. 3. *A*, same specimen and section thickness. In this case the section was mounted without treatment. "Knife marks" and occasional slight folds represent the only artifacts of sectioning in this relatively large field. Approximately × 1400. *B*, same specimen, section thickness, and treatment as in *A*. Note extreme fineness of nuclear membrane in comparison with Fig. 2. Note also individual cell membranes for adjacent cells and multitude of cytoplasmic structures. The heavy line at the top right-hand corner is a fold. The more diffuse broad band in the lower left-hand corner is a defect introduced by the dense granule at the inner end. Approximately × 3100.

marks," occasional folds, and local deformations caused by different cutting properties of parts of the tissue, especially near large blood vessels. Most of the artifacts do not interfere with the correct interpretation of the images. However, there is, as yet, no control on the artifacts introduced by the fixation and embedding of the tissue. Preliminary trials of the technique on other material have given varying degrees of success. They indicate the need to adjust the embedding to the hardness of the tissue being cut, and to employ sharper knives.

SERIAL SECTIONS

When a Spencer rotary microtome (No. 820) is modified according to the method described by Pease and Baker (*3*) to cut sections of the thickness suitable for examination by means of the electron microscope, serial sections cannot be cut in the conventional way. However, the necessity of having serial sections is apparent when one considers that more than 100 sections are required to survey a single cell. Furthermore, serial sections provide the most direct indication of the artifacts introduced by the sectioning and mounting procedures.

The failure of the Pease and Baker technique to provide serial sections is a result of the extreme flexibility of the thin sections, which allows them to be rolled and folded by the slightest resistance encountered after being cut. Subsequent spreading and flattening of a number of consecutive sections on a liquid surface are exceedingly difficult, if not impossible, and are further obstructed by the destruction and adhesions produced by removing the dry group of sections from the edge of the blade. In the present work these difficulties have been largely overcome by bringing a liquid surface to the cutting edge of the knife, and by allowing the sections to float to the surface of the liquid as they are cut. When the surface tension and level of the liquid are adjusted properly, the sections

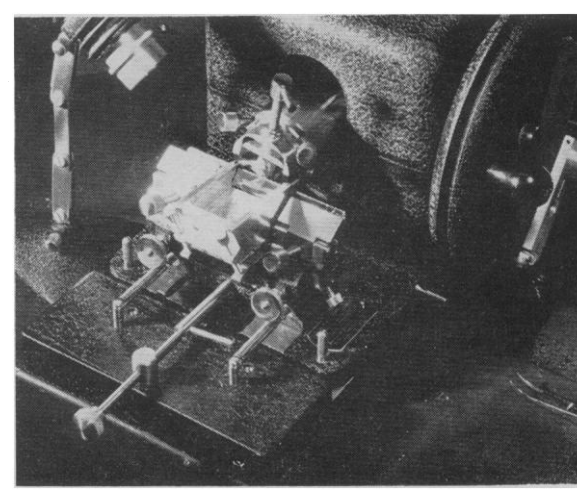

FIG. 4. Photograph of the liquid reservoir as attached for actual cutting. Also shown is a modification of the microtome, which permits a more critical initial adjustment of the knife. The illuminator shown in this photograph was found to introduce intolerable thermal drifting and is no longer used.

are kept full-extended, and long ribbons of sections are obtained ready for mounting (*2*).

The liquid is contained in a reservoir, which is clamped on the knife between its supports (Fig. 4). It consists of a trough machined from aluminum. The knife acts as the end wall of the reservoir, the seal between it and the trough being provided by a gasket fitted into a groove around the end of the trough. Two levers with hooked ends that pass under the knife, and two screws acting on the opposite ends of the levers, comprise the clamping mechanism. It is obvious that the reservoir must extend somewhat above the edge of the knife, and that the seal between the reservoir and the knife must reach the edge. As might be expected, this leads to some impairment of the edge at the point where the gasket is pressed against it, but the defect introduced is slight and is removed by normal sharpening.

The specimens used in the present work were doubly embedded in collodion and paraffin. Forty percent alcohol proved to be the most generally satisfactory liquid, though it was found desirable in many cases to adjust the concentration. The optimum concentration for a given situation has always fallen within the range 20–60 percent. Dioxane in water in concentrations of less than 50 percent has been found best in some cases, but some doubt remains as to the effect of the dioxane on the sections. Some corrosion-retarding agent should be used in the liquid, particularly in the case of dioxane in water. The addition of one part per thousand of chromglucasate has been very satisfactory for this purpose. The criteria for the adjustment of the concentration of the liquid have been developed from continuous observation of the cutting through a stereoscopic microscope (×27) and from correlation with the quality of the sections, as judged in the light and electron microscopes (×400 and ×1000–5000), respectively.

Another important parameter in the adjustment of the liquid is its level relative to the edge. It has been found empirically that the best sections are obtained when the level is maintained as high as possible. The limiting condition in this regard is that the block must remain dry for a sufficiently long series of sections. It is further determined by a number of factors relating to the nature of the tissue, its fixation, its embedding, the sharpness and cleanliness of the knife, the clearance angle, and the nature of the liquid. The correct level is found by raising the height of the liquid slowly as sections are being cut and observed at a magnification of at least nine times. When the level is slightly too high, a small area will be wet by the liquid. Moreover, the wet area will increase with each successive cut. It is a simple matter to achieve this condition and then to lower the level until the wet area diminishes and disappears. If, by accident, the entire face of the block gets wet, the situation can be corrected by lowering the level of the liquid approximately 1 mm below the edge of the blade until the sections appear dry. The correction can be hurried by drying the back of the knife and the face of the block with absorbent cotton.

This system of collecting specimens as they are cut has fulfilled very satisfactorily its intended purpose of providing serial sections. It has also made two unexpected but welcome contributions to the program, in that it provided sensitive criteria for detecting irregularities in the performance of the advancing mechanism of the microtome and for judging the quality of the sections even before they are examined in the electron microscope. It is the practice in this laboratory to examine sections at magnifications of nine times or twenty-seven times as they are cut. It was soon realized that for a given knife, block, liquid, etc., the length of a floating section (measured in the direction of cutting) or, conversely, the *apparent* compression is a sensitive criterion for the thickness of the layer removed from the block—that is, the magnitude of the advance. Since, by this means, any irregularities in the magnitude of successive advances (as small as 5 percent) are immediately observable, it is a relatively simple matter to identify and eliminate the various sources (*2*).

Continuous observation of the sections as they are cut and correlation with the results obtained in the electron microscope showed that satisfactory sections appear on the surface of the liquid instantaneously and completely extended, as each cut is made. Moreover, they appear quite transparent and almost invisible. On the other hand, an unsatisfactory section seems to slide slowly to the surface from a folded condition near the edge of the knife, and it never attains the transparency of the satisfactory sections. It has also been found that the height to which the liquid can be raised without wetting the block is a good criterion of section quality. No good sections have been obtained when difficulty has been encountered in achieving a plane or convex meniscus, though it is not to be implied that such a meniscus is necessary to successful cutting.

References

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