SOME EFFECTS OF THE MICROTOME KNIFE AND ELECTRON BEAM ON METHACRYLATE-EMBEDDED THIN SECTIONS*

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PLATES 4 TO 7

Hillier and Cannan (1) and Watson (2) showed that sublimation of methacrylate occurs in the electron beam. Recently, Williams and Kallman (3), employing the techniques of shadow casting and stereoscopy, studied apparent discrepancies encountered in serial sections and suggested that they resulted from sublimation of methacrylate and loss of tissue between sections. The purpose of this preliminary report is to describe a method of visualizing sections at low electron beam intensity. Certain types of distortion produced by the electron beam and by the microtome knife will be illustrated and discussed.

MATERIALS AND METHODS

Tissues were fixed in 1 per cent osmium tetroxide buffered at pH 7.4, according to the method of Palade (4), or in 4 per cent neutral formalin. After dehydration in graded ethyl alcohols they were embedded in pure butyl, or in combinations of butyl and methyl, methacrylate. The majority of sections were cut on Porter-Blum type microtomes (5) equipped with glass knives (6),1 and picked up from an acetone-water mixture on formvar coated copper grids.

An RCA type EMU-2E electron microscope with a biased gun and a 20 mil condenser aperture was employed. In order to examine the effects of the electron beam, micrographs were made in the following manner. The condenser current was set at maximum and the gun was only partially saturated. Although no image was visible on the fluorescent screen, a satisfactory image could be obtained on the photographic plate by exposure from 1 to 7 minutes at a magnification of 2000. (For convenience these conditions will be referred to hereafter as low beam.) After saturating the gun, a second plate was exposed for 40 to 50 seconds (medium beam). The current in the condenser lens was then reduced and the specimen was subjected for a few seconds to the beam intensity customarily employed. The current in the condenser lens was again set at maximum and several plates were exposed for periods varying between 12 and 20 seconds (post-high beam). The negatives obtained by exposure to low, medium, and post-high beam intensities had approximately the same density. The images therefore reflected the appearances of the section under somewhat similar conditions of micrography. The current was not changed in either the projector or the objective lens at any time during these maneuvers.

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1 The authors wish to thank Dr. Keith Porter and Dr. Robley C. Williams for their kindness in cutting some of the sections. Although distortion was minimal in their sections, it resembled the types to be described below.

RESULTS

Fig. 1 illustrates a section of chicken chorioallantoic membrane fixed for 5 minutes in osmium tetroxide. The negative was exposed for 180 seconds at low beam intensity. An entodermal cell occupies the lower part of the field. Knife scratches indicate the direction of cutting. Wide bands of differing size cross the field in a direction parallel to the knife edge. Superimposed on these are bands of alternating thick and thin zones with a periodicity averaging 0.2 micron. The thin zones frequently merge. A narrow fold in the section crosses the upper left corner. Fig. 2 illustrates the same field micrographed at medium beam intensity with an exposure of 40 seconds. The density of the methacrylate relative to the tissue has decreased. The wide bands and knife scratches exhibit less well-defined margins. Although the narrow bands persist within the tissue, they have largely disappeared from the remainder of the field. The fold at the corner is wider. In the post-high beam micrograph (Fig. 3), exposed for 16 seconds, the density of the methacrylate has further diminished and only a suggestion remains of the fold, the wide bands, and deepest knife scratches. Except within the cytoplasmic inclusion lying close to the cell border, the narrow banding has been obliterated. It should be noted that the density of this inclusion reflects the relative duration of exposure in printing the negative. Very little structure would be visible if the print of Fig. 1 had been exposed a sufficient length of time to render the inclusion as dense as it appears in Fig. 3.

The actual density of a considerably thinner section micrographed with the low beam technique is illustrated by Fig. 4. The tissue is the mesoderm of chorioallantoic membrane fixed for 5 minutes in osmium tetroxide. Knife scratches run diagonally. Compared to the preceding section, thick and thin bands are fewer in number, less clearly demarcated, and more variable in periodicity. One particularly thin zone is indicated by the arrow; to its left is a single thick band. In the post-high beam micrograph (Fig. 5), the density of the methacrylate has markedly diminished and no knife scratches or bands are visible. At the site of the thin zone previously mentioned, the limiting membrane and adjacent cytoplasm of a cell are missing.

Comparison of low and post-high beam micrographs revealed that changes in the shape and orientation of tissue components frequently had taken place. In order to study in detail the distortion which may result from impact of the electron beam, the hexagonal array of myofilaments was examined. Fig. 6 illustrates a cross-section of canine heart muscle (left ventricle) fixed for 1 hour in osmium tetroxide. The plate was exposed for 7 minutes. Myofibrils are present in the lower two-thirds of the field. Dense, irregularly shaped particles are seen within the sarcoplasm. The sarcolemma runs horizontally and above it is the basement membrane of a capillary. The capillary lumen occupying the upper border of the illustration is presumably filled with methacrylate, indicating the relative density of the embedding plastic. The low density between the
lamellae of the basement membrane, as well as that between the myofilaments, would suggest that little if any plastic had infiltrated these areas. Fig. 7 illustrates the same field micrographed with the post-high beam technique. The negative was exposed for 15 seconds. There has been sublimation of methacrylate and general shrinkage, most marked in the myofibrils. The spacing of the myofilaments has been disturbed, some filaments appearing to have been tilted and therefore to overlap. The dense particles in the sarcoplasm are less numerous. The poor definition and discontinuity of the sarcolemmic membranes may result not only from tilting but also from direct injury. Another field from the same two negatives is shown in Figs. 8 and 9. Comparison again reveals loss of particulate components from the sarcoplasm. The hexagonal array of myofilaments has been nearly obliterated, except in one area near the top and another in the middle third of the field. The average spacing of the filaments in these areas is indicated for both Fig. 8 and Fig. 9 by Text-fig. 1. It is evident that the distortion produced by the electron beam differs in each axis measured. The number below the hexagons representing the average length of the sides reveals a total shrinkage of approximately 7 per cent.

In order to study the effect of the electron beam on formalin-fixed tissue, choroioclantoic membranes were micrographed with medium and post-high beam techniques (Figs. 10 to 15). Fig. 10 shows part of the cytoplasm of an ectodermal cell infected with vaccinia virus and fixed for 1 hour in 4 per cent neutral formalin. Several membranes are present in the lower half of the field. Below and to the left of them is an irregular, moderately dense cytoplasmic

Text-Fig. 1. The spacing of the hexagonal arrays of myofilaments illustrated in Figs. 8 and 9. The numbers below the diagrams are the average values of the sides. They indicate that exposure to the beam resulted in an over-all shrinkage. The arrow shows the direction of cutting.
inclusion. In the upper left corner a vaccinia particle exhibits a structure and density similar to that encountered after fixation with osmium tetroxide (7). Scattered through the cytoplasm are small particulate components which have been described by several investigators (8-11). Fig. 11 is a post-high beam micrograph of the same field. The inclusion is less dense. The cytoplasmic membranes and sharply defined structure of the virus have been obliterated. Although some of the small particulate components appear to be unaffected, others either exhibit diffuse outlines or have disappeared. Fig. 12 shows a cluster of vaccinia viral particles in the cytoplasm of a cell fixed for 4 hours and micrographed with the medium beam technique. In the post-high beam micrograph (Fig. 13) the particles are less dense and their structure is poorly defined. Figs. 14 and 15 illustrate medium and post-high beam micrographs of part of two mesodermal cells also fixed for 4 hours. To the right of the nucleus at the upper border are two cytoplasmic inclusions. After subjection to high beam intensity both inclusions exhibit indistinct margins and the material comprising one of them appears much less dense. Below the nucleus in the lower third of the field several other cytoplasmic inclusions show similar changes. Selective loss of particulate components in the cytoplasm and nuclei has occurred. Comparison of the dimensions of the lower nucleus in the two micrographs reveals that although there has been no change along the vertical axis, there has been slight shrinkage along the horizontal axis. Four inclusions in the lower third of the field have swelled under impact of the beam, whereas two (in the lower left corner) are unaltered.

DISCUSSION

Two types of bands were illustrated in Fig. 1. Those with long periodicity crossing the entire field without interruption and varying considerably in thickness are believed to result from vibration encountered during sectioning. The bands of shorter periodicity differed from these sufficiently to suggest another mode of origin. They occupied large areas in some sections and small areas in others. Not infrequently they were confined to a localized structure, such as an erythrocyte or a cytoplasmic inclusion. In general, dense and apparently rigid structures were more susceptible. For example, normal mouse lungs cut smoothly, whereas consolidated lungs were difficult to section without banding. The quality of the knife edge also was a factor. As pointed out by Williams and Kallman (3), in chipped-out regions of the knife the effective included angle of the cutting "edge" would approximate 90° rather than 45°. Such nicks appeared to plow through the section and frequently to produce a narrow, localized area of banding on each side. Banding was usually pronounced when the nicks were numerous. The branched appearance of these

2 The term "inclusion" is used to describe a variety of aggregated materials encountered in normal as well as diseased cells. No relation to viral inclusion bodies is implied.
bands and their uneven distribution, predilection for certain tissues and cell components, and association with nicks in the knife—all suggest that they do not result simply from mechanical vibration. It is more likely that the methacrylate and embedded tissue were distorted by impact of the knife and piled up in front of the cutting edge. When resistance of the structures to cleavage equalled the restoring force of their elasticity, the knife passed through the zone of distortion and produced a thick band. The process was then repeated, the thickness and periodicity of the bands reflecting the state of the knife edge as well as the toughness and elasticity of the tissue. The term "periodic distortion" seemed appropriate to describe this phenomenon and to distinguish it from bands of longer periodicity produced by vibration, as well as from the wrinkling or rippling of short (30 m$^3$) periodicity described by Williams and Kallman (3).

Disappearance of the banding in the electron beam resulted, in part, from sublimation of the methacrylate. More methacrylate appeared to sublime from the thick areas, presumably because absorption of energy from the electrons is greater in these areas. Although differential sublimation would tend to smooth out the plastic, it would not efface the banding within tissues. Changes in size, shape, and orientation of structures during bombardment at ordinary beam intensities suggested that softening of the methacrylate also occurred. If tissue components distorted by sectioning were elastic they would then tend to assume their original shape, whereas inelastic structures, such as certain cytoplasmic inclusions, would continue to exhibit the thick and thin zones. Although such an hypothesis cannot be proved, it does help to explain why the periodic distortion is obliterated in some tissue components and persists in others.

Distortion caused by the knife was manifested not only by periodic banding, but also by characteristic deformation of structure. Vaccinia and fowlpox viruses at one stage of development have invariably appeared ellipsoidal with major axes parallel to the knife edge. Such constant orientation can best be explained by assuming that the virus was spherical before sectioning. In a separate communication (12), consecutive serial sections of vaccinia virus were illustrated. Comparison of sections I and II (Fig. 2 of the paper) reveals that in the former, where deformation is more marked, the major axes of the ellipsoidal particles have lengthened, whereas the minor axes have shortened. These and other serial sections have shown that the lengths of the major and

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3 The interpretation of sections shadowed before examination is complicated by the fact that extensive sublimation of methacrylate occurs in the beam despite the presence of metal on the surface. Comparison of low and post-high beam micrographs revealed shrinkage of the tissue with lengthening of pre-existing shadows and the introduction of clefts which resembled shadows.

4 It is evident from this observation that the opacity of the methacrylate under the usual conditions of beam intensity is not proportional to the thickness of the section.
minor axes are inversely proportional. Although the small size or variable shape of a majority of tissue components makes accurate determination of distortion in serial sections difficult, deformation similar to that described above has been repeatedly encountered in nuclei of cells. The observation by Williams and Kallman (3) that "cut sections are no wider than the block from which they are cut" appears to conflict with the fact that structures showing shortening in the axis perpendicular to the knife edge exhibit elongation in the axis parallel to it. Actually, however, deformation can occur locally without widening of the section. In the illustrations of vaccinia virus previously referred to (12), it is evident that deformation has increased the spacing of the particles in a direction parallel to the knife edge. Yet examination of the original negatives, which show a considerably larger area of the section, reveals that the distance separating two points on each side of the viral cluster has remained constant. In other words, the widening is confined to the aggregate of virus and is not reflected in the field as a whole. A similar phenomenon is illustrated in a paper dealing with serial sections of *Porthetria dispar* virus (13).

Although formalin has proved to be an adequate fixative for the electron microscopy of relatively thick sections from which the methacrylate was removed by a solvent (14), it has proved to be inadequate for the preservation of structure in ultrathin sections examined with the embedding plastic in place. Only part of this discrepancy can be explained by the severe distortion of fine structure accompanying the use of solvents. The data presented herein suggest that methacrylate-embedded, formalin-fixed tissue is unstable and that incineration or sublimation of a variety of tissue components results from impact of the electron beam at the intensities customarily employed. It is pertinent that Draper and Hodge (15) observed selective micro-incineration of the Z and M bands in formalin-fixed muscle fragments bombarded by the electron beam and noted that fixation with osmium tetroxide resulted in greater stability. Since our preliminary observations indicate that, under conditions of low beam intensity, the density of certain structures is similar for both fixatives, it seems unlikely that the greater stability resulting from fixation with osmium tetroxide directly reflects actual deposition of the metal.

In order to study contamination, sections were exposed to the electron beam at cross-over for periods up to 1 hour. Through-focus micrographs were taken at regular intervals. Contamination by a substance showing no structure when examined at a resolution of 20 to 30 Å was manifested by progressive darkening of the field with gradual loss of contrast in the section. Although contamination was more rapid when the beam struck the copper grid supporting the section, molecular arrays similar to those illustrated for the polyhedron enclosing *Bombyx mori* virus (16) were clearly resolved, even adjacent to the metal, after exposure for 16 minutes. High resolution was seriously affected only when structures of low density were subjected to intense bombardment for considerable periods of time.
It should not be inferred that the types of distortion discussed above necessarily occur in all sections. This preliminary report has presented a new technique for the examination of specimens at low electron beam intensity and has demonstrated that micrographs of thin sections which have been exposed to the usual beam intensities may not accurately reflect the morphology of the tissue embedded in the block.

SUMMARY

A technique for the examination of specimens at low electron beam intensity has been presented. Sections micrographed with this technique showed numerous knife scratches and frequently contained bands running parallel to the knife edge. Banding with an average spacing of 0.2 μ appeared to result from periodic distortion produced by impact of the knife. At the beam intensities customarily employed, differential sublimation and probably flow of the methacrylate resulted in obliteration of the bands and all but the deepest knife scratches. In addition, changes in the size, shape, and orientation of certain structures were noted. Artifacts resulting from incineration or sublimation of tissue components fixed in formalin were illustrated, and the suggestion was made that such instability to the electron beam accounted in part for the differences observed in osmium- and formalin-fixed tissues. The deformation revealed in serial sections was discussed, and it was pointed out that shortening in the axis perpendicular to the knife edge was associated with elongation in the axis parallel to the cutting edge, the elongation usually occurring locally without change in the width of the section. It was noted that the material causing contamination of the surface of sections during examination exhibited no structure but caused progressive loss of contrast.

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REFERENCES

EXPLANATION OF PLATES

PLATE 4

FIG. 1. A micrograph taken at low electron beam intensity with an exposure of 180 seconds. Scratches traverse the field diagonally and indicate the orientation of the knife with respect to the section. Wide bands of differing thickness run in a direction parallel to the knife edge. Superimposed on these and parallel to them are thick and thin zones with an average periodicity of 0.2 μ. A narrow fold in the section crosses the upper left corner. × 9000.

FIG. 2. The same field micrographed at medium beam intensity with an exposure of 40 seconds. Although differential sublimation of the methacrylate has begun to smooth out the contours of the section, the banding remains clearly defined within the cytoplasm of the cell occupying the lower border of the illustration. × 9000.

FIG. 3. The same field micrographed after subjecting the section to the beam intensity usually employed. The narrow bands have been entirely obliterated, except within the cytoplasmic inclusion near the free border of the cell. The deepest knife scratches remain but are poorly defined. The loss of density and widening of the fold in the upper left corner suggest that differential sublimation and flow of methacrylate has occurred. × 9000.
(Morgan et al.: Methacrylate-embedded thin sections)
PLATE 5

Fig. 4. A low beam micrograph printed to show the actual density of the methacrylate. The section was considerably thinner than the one illustrated in the preceding plate. Bands of variable length and periodicity traverse the field in a direction vertical to the knife marks. Within one particularly thin band a defect in the section is indicated by an arrow. × 14,000.

Fig. 5. A post-high beam micrograph of the same field. Sublimation of methacrylate has markedly increased the contrast between the tissue and the embedding plastic. The defect is reflected by discontinuity of the cell membrane and loss of adjacent cytoplasmic components. (The very fine, irregular, thread-like lines of diminished density which traverse the field appear in the formvar when the specimen grid is slightly bent during handling.) × 14,000.
(Morgan et al.: Methacrylate-embedded thin sections)
FIG. 6. A low beam micrograph of cross-sectioned cardiac muscle. The negative was exposed for 7 minutes. A myofibril with sharply defined myofilaments occupies the lower two-thirds of the field. At the upper border is the lumen of a capillary. \( \times 44,000 \).

FIG. 7. A post-high beam micrograph of the same field. The membranes composing the sarclemma are indistinct and discontinuous. Particulate components of the sarcoplasm have disappeared. The myofibrils have shrunk with displacement of myofilaments. \( \times 44,000 \).

FIG. 8. A different area of the negative used for Fig. 6. Knife marks traverse the field horizontally and several bands of periodic distortion run vertically. A myofibril occupies most of the illustration. \( \times 44,000 \).

FIG. 9. The same field from the post-high beam negative. The spacing of myofilaments has been altered. \( \times 44,000 \).
(Morgan et al.: Methacrylate-embedded thin sections)
PLATE 7

Fig. 10. A medium beam micrograph of part of an epithelial cell fixed for one hour in neutral 4 per cent formalin. Laminated membranes traverse the lower half of the field. A vaccinia viral particle is present in the upper left corner. $\times 33,000$.

Fig. 11. A post-high beam micrograph showing loss of density and definition of the membranes and virus. The inclusion seen in the lower right corner is also less dense. Although many cytoplasmic particles have disappeared, others seem to have been unaffected. $\times 33,000$.

Figs. 12 and 13. Low and medium beam micrographs of a cluster of intracytoplasmic viral particles fixed for 4 hours in neutral formalin. $\times 34,000$.

Figs. 14 and 15. The effect of the electron beam on two formalin-fixed mesodermal cells. Selective destruction of intracytoplasmic and intranuclear particulate components is apparent. Several inclusions have become less opaque. The size of many of the inclusions, as well as of the nucleus in the lower half of the field, has been altered. $\times 13,000$. 