REDUCTION OF HEATING ARTIFACTS IN THIN SECTIONS EXAMINED IN THE ELECTRON MICROSCOPE

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It has been demonstrated by Morgan (1) that at the beam intensities normally used by electron microscopists, there can be appreciable distortion of small structures in tissue sections and a complete loss of some of these structures. That there is also a substantial loss of embedding material from sections is well established (2). In the present work we investigate these phenomena and decribe a technique for reducing the destructive effects of the electron beam.

Materials and Methods

Intact tissues were fixed in 1 per cent OsO_4 buffered in veronal-acetate either with or without the addition of sucrose. Isolated mitochondria were fixed as pellets in unbuffered 1 per cent OsO_4 for 16 hours. All preparations were dehydrated in ethyl alcohol and embedded in butyl methacrylate. A Porter-Blum microtome provided with a glass knife and collection trough containing 10 per cent acetone was used. Sections were cut to a standard thickness giving definite silver interference colors from which traces of yellow were absent. The sections were mounted in various ways described in the text on grids bearing either carbon films or carbon nets (3–6).

All micrographs except Figs. 7 and 8 were made with a Siemens Elmiskop I using the double focussing condenser with a 500 μ condenser aperture. The accelerating voltage was 60 kv. and a 50 μ objective aperture was used. Figs. 7 and 8 were made with an RCA type EMU-2A microscope equipped with a 250 μ condenser aperture and a 25 μ objective aperture. It was the general practice to use beam intensities no higher than necessary for focussing.

RESULTS

Sections of embedded tissue may be micrographed with substantially greater contrast when mounted without substrate than when in contact with a supporting film (3–6). In the author's experience, the addition of even a very thin substrate of formvar or collodion, for example, reduces the contrast considerably. What is not immediately clear is why so thin a layer of substrate should have such a marked effect. Three possibilities suggest themselves: (*a*) random scattering in the substrate is responsible; (*b*) there exists some hitherto unobserved contrast phenomenon at the exposed surface of the section which is

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eliminated by covering with substrate; or (*c*) loss of embedding material is greater when both surfaces of the section are exposed than when one is covered with substrate. The following experiment and other observations to be presented favor the last of these possibilities.

A section was mounted on the back side of a carbon-filmed Athene[1] grid so that most of the section was held away from the carbon film. At some points, however, the section touched the carbon in the central portion of a grid opening but was separated from it near the edges of the opening by the intervening copper mesh. Where the section parted from the carbon film there was a marked and abrupt increase in contrast (Figs. 1 and 2) despite the fact that the carbon film was still present in the optical path. This clearly eliminates the first possibility that the increase in contrast observed in unsupported sections is due to absence of optical interference by the supporting film. Further examination of the micrograph reduces, by its strong support for the third, the likelihood that the second possibility is a major contributor. It will be noted (Figs. 1 and 2) that the intertissue regions of the unsupported part of the section are paler than are corresponding parts of the supported portion while the converse is true of regions containing tissue. Two effects are suggested to explain this. The unsupported part of the section is tilted at a steep angle to the plane of the grid as it leaves the carbon film to pass over the grid wire; thus for equal film thickness, areas in the unsupported portion being viewed obliquely will present greater mass in the optical path and will therefore appear darker than corresponding areas in the supported portion. Hence the tissue-bearing areas exhibit higher image density in the unsupported portion. The intertissue regions, however, in our view have lost a considerable amount of embedding material and appear paler in the unsupported portion despite the fact that they are viewed obliquely. We are led to conclude that a part of the higher contrast of unsupported sections is due to greater loss of embedding material when both sides of the section are exposed. This conclusion disagrees somewhat with that of Williams and Kallman (2) who reported that sandwiching of sections between films of formvar had little effect on the rate of sublimation.

Williams and Kallman concluded that discrepancies they observed between details in adjacent serial sections arose from actual losses of material dislodged during sectioning and from effects of sublimation. Morgan *et al* (1), previously referred to, compared micrographs of areas of sections exposed only to extremely low beam intensities to micrographs of the same areas at normal or intermediate beam intensities. It was quite evident from their micrographs that exposure to normal or intermediate beam intensities could introduce substantial distortion of details of tissue and could even remove completely certain structures. The electron beam was especially effective in removing tissue from sections of formalin-fixed material and less so when OsO_4 was the fixative.

[1] Manufactured by Smethurst High-Light, Ltd., Sidcot Heaton, Bolton, Lancashire, England.

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On the basis of these findings and the observations described earlier in this paper it was thought that sandwiching of the section might minimize loss of embedding material and might reduce distortion of the tissue. A carbon net (3–6) was covered with a thin layer of collodion containing fewer and smaller holes than those in the net. The section was mounted on this and in turn covered with another layer of collodion without holes. Thus the section was mounted in such a way that part of it was sandwiched between two layers of collodion in some areas while immediately adjacent were areas with one side exposed through a hole in one of the collodion films. Cross-sections of muscle fibrils from rat diaphragm were chosen for examination because of the ease of detecting disruption of order inherent in the muscle and because this particular material was found difficult to micrograph at high resolution when prepared by ordinary methods. In the micrographs (Figs. 3 and 4) the transition between the sandwiched and unsandwiched zones is not distinct; however, the sandwiched zones (Fig. 4) appeared somewhat darker[2]. The muscle fibrils are slightly separated from one another, the space between being occupied mainly by mitochondria, elements of the endoplasmic reticulum and by relatively dense, round particles. The most striking difference between the sandwiched and unsandwiched portions lies in the appearance of the interfibrillar material. Where the section is unsandwiched (Fig. 3) the interfibrillar material, particularly the endoplasmic reticulum and the small particles, is indistinct and poorly delineated and in some areas there actually appears to be less material present. The improvement within the muscle fibrils where sandwiched (Fig. 4) is less pronounced although such areas look somewhat "cleaner" and more orderly. This generally more satisfactory appearance of sandwiched sections has also been observed in a number of other tissues including intestinal epithelium, connective tissue and muscle of the leech, rat enamel epithelium, and rabbit bone marrow.

Less subtile than the improved appearance of muscle is the better preservation of certain dense objects such as secretion granules, lipid inclusions, and isolated mitochondria. These specimens tend to contract under the electron beam so that they may separate from the surrounding material or develop holes. Such separations or holes are characterized by markedly lower density than the rest of the section. When mounted on carbon which does not deform readily, presumably the contraction is effected by local peeling of the section from the substrate. If the section is supported on formvar or collodion, contraction without peeling can take place more easily. These effects of contraction with or without peeling are much reduced by sandwiching. This is illustrated by the appearance of unsandwiched and sandwiched sections of granules in a cell of rabbit bone marrow (Figs. 5 and 6) and of isolated mitochondria (Figs. 7 and 8). These particular sections were supported on continuous carbon films, the sandwiched ones being in addition covered with a film of collodion. In both

[2] The micrograph was printed to compensate for this.

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cases where the section is unsandwiched (on the left), holes of low density and with sharp edges are present in some of the elements and all of them display an irregular somewhat diffuse outline which is absent in the sandwiched sections (on the right). Shrinkage of this sort, even where holes are not formed, often masks details that might otherwise be seen. Thus, in granules of bone marrow cells (Fig. 6) differences exist between the interior and the cortex of the granules that cannot readily be discerned in the unsandwiched section (Fig. 5).

Certain structures, as Morgan showed, are rather labile and tend to disappear from sections exposed to the electron beam. A particularly striking example was found in certain connective tissue fibrils in the region of the gut wall of the leech. In unsandwiched sections (Fig. 9) these fibrils are almost invisible on the microscope viewing screen and rather sparse in micrographs, whereas in sandwiched sections the situation is quite the contrary (Fig. 10). The micrograph of the unsandwiched section is printed at high contrast in order to show the fibrils at all. It will be noted that the walls of what may be two cell processes are very dense. In the micrograph of the sandwiched section printed on much softer paper, while the fibrils stand out distinctly, the wall of a similar process is much less dense than in the first micrograph. This suggests that material present in the fibrils of the sandwiched section was absent from the unsandwiched preparation and was removed by exposure to the electron beam.

It seems reasonable to suppose that the presence of added layers of scattering material as introduced by this technique will reduce the ultimate resolution possible with a given section. Fig. 11 shows the membrane of a muscle cell of the leech in which points can be found where the spacing of the paired elements is 30 to 50 A center-to-center. Judging by the clear separation even at these points, the resolution of biological structure is at least 25 A in this micrograph. Thus satisfactory resolutions for most work can be obtained with sandwiched material.

DISCUSSION

The principle defect in the use of sandwiching lies in the reduction of contrast, already at a premium, by perhaps one grade of photographic paper. The difficulty here is that the presence of grain, made visible by phase contrast (7), limits the amount by which contrast in the image can be increased photographically. The grain has two properties which are of interest in the present discussion: its dimensions or coarseness and its contrast relative to that of the structures one wishes to reveal. The coarseness decreases with decreasing distance from focus and the grain disappears entirely at focus. Unfortunately, contrast of structural details is much lower at focus than it is away from it. The contrast of the lightest to the darkest points in the grain depends in a complex way on the density and thickness of the film producing the grain. The effect of sandwiching is to reduce the contrast of dense structural details against the background with, we believe, little change in the grain. This means that in micrographs of identical sections printed for the same image contrast the grain will be more notice-

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able in the sandwiched section. The increase in "graininess" will be proportionate to the increase in contrast required to bring out details of structure. The best procedure to minimize these effects is to support the section, sandwiched between thin films of collodion or formvar, on carbon or chromium nets.

Precisely why covering both sides of the section improves the appearance of the embedded tissue is not entirely clear. We have presented evidence which supports, although not conclusively, the idea that the presence of covering films reduces loss of embedding material in the electron beam. The covering films may serve to reduce somewhat the temperature of the section by providing a greater cross-section for thermal conduction. However, it does not seem likely that the temperature lowering is sufficient to be very effective. There is no doubt that embedding material is lost even from sandwiched sections because some clearing of the section can be seen on first exposure of an area to the electron beam. This clearing occurs less rapidly than in the case of sections with only one side exposed. Butyl methacrylate polymer softens and even becomes liquid at relatively low temperature (*ca.* 100°C.) A heated section with one side exposed to the vacuum will present an interface at which forces of surface tension may produce small-scale migration. When the section is covered on both sides by material of higher melting point, these surface tension effects are much weakened. We believe that this may account in part for the improvement resulting from sandwiching which is demonstrated here. In some cases sandwiching reduces loss of components of the tissue. We are unable to account for this unless perhaps such material is loosely bound and flies off by electrostatic repulsion from the charged section when the methacrylate softens. Such material may be held in place by the covering film.

SUMMARY

Certain phenomena affecting contrast obtained from tissue sections with the electron microscope have been investigated and a technique is described for reducing destruction by the electron beam of fine details in sections. It has been concluded that loss of embedding material is slightly higher at exposed surfaces of sections than it is at surfaces covered by substrate film. Covering of both surfaces of sections with thin films of formvar, collodion, or carbon materially improves the general appearance, reduces distortion, and sometimes reduces loss of tissue mass from the section as result of exposure to the electron beam. This improvement is considered to result from the relatively high melting-point of the covering films which serve to eliminate or reduce surface-tension or other forces operating in methacrylate softened by the electron beam.

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EXPLANATION OF PLATES

PLATE 313

FIGS. 1 and 2. Section of enamel epithelium of the rat. The section and the carbon substrate film were mounted on opposite sides of a copper grid, 35 μ in thickness. In some areas the two films were in contact, while in others they were held apart by the intervening grid wire. In the center of each picture the dense, horizontal line is the boundary between two such areas. The section and carbon film are in contact in the upper half of each picture and are separated in the lower half. By following along the margin of the grid opening, points were found where holes had developed in the unsupported part of the section. Through such holes the carbon film could be detected by the presence of small particles of dirt on it. Thus it is known that the carbon film was intact and present in regions where it was not in contact with the section.

There is a sharp increase in contrast where the section leaves the carbon film. This is due to greater image density in the tissue regions and lower image density in the intertissue regions. Greater density of the tissue can be accounted for by the fact that as the section leaves the carbon film it is tilted at a relatively steep angle to the plane of the grid in order to pass over the grid wire. Thus for areas of equal thickness the tilted portion presents greater mass in the optical path than does the untilted area in contact with the carbon film. The lower density of the intertissue regions is probably due to greater loss of embedding material where two sides of the section are exposed.

Fig. 1, × 30,000; Fig. 2, × 60,000.

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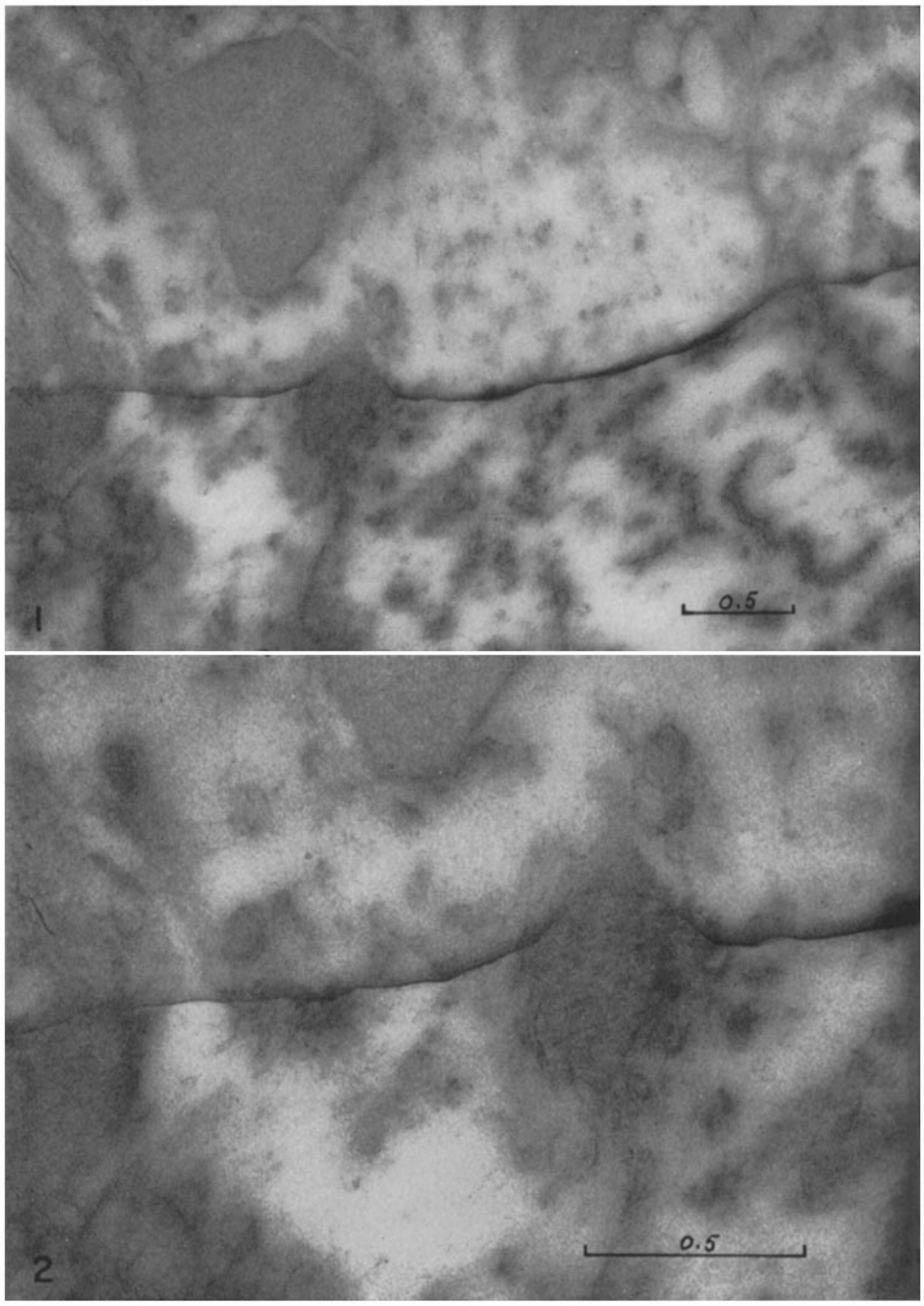

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PLATE 314

FIGS. 3 and 4. A single micrograph of a section of muscle from rat diaphragm. The section was sandwiched between collodion films, one of them continuous and the other containing relatively large holes. A carbon net was used as support. The micrograph was made at the edge of a hole in one of the collodion films so that the upper half is unsandwiched and the lower half, sandwiched, and the two halves of the micrograph were printed to approximately equal density.

The most noticeable effect of the sandwiching (Fig. 4) is the prevention of the severe retraction distortion of the interfibrillar regions (Fig. 3). This distortion is so pronounced in the unsandwiched area that both the round, dense particles (*p*) and the elements of the endoplasmic reticulum (*er*) present between the fibrils are very indistinct. Membranes of mitochondria (*m*) suffer from this distortion to a lesser degree, but these and the organization of muscle filaments within the fibrils are somewhat clearer in the sandwiched area. A number of micrographs of different areas of sections of this tissue consistently showed the same kind of distortion in the unsandwiched regions and its elimination by sandwiching. × 110,000.

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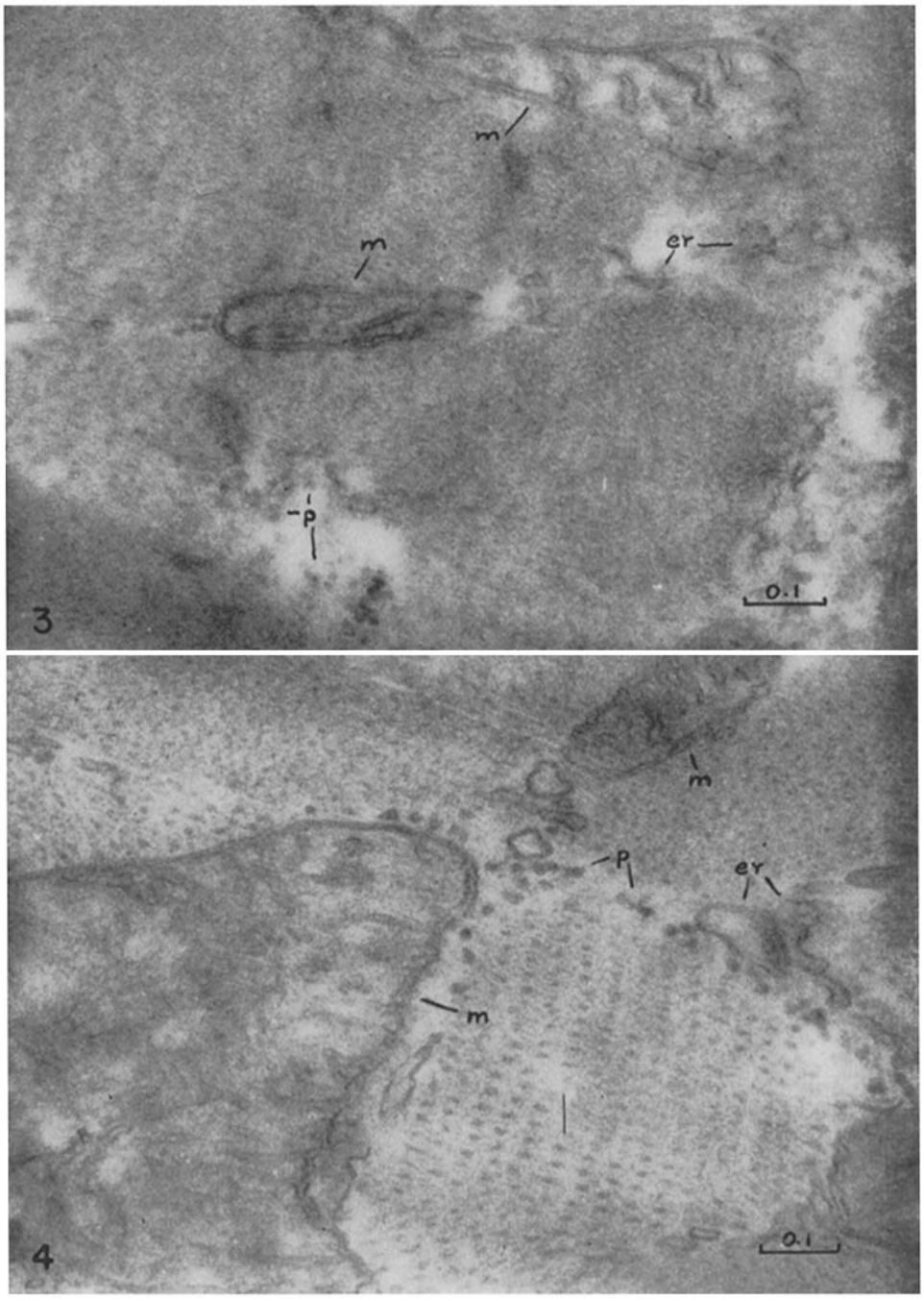

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A comparison of unsandwiched (Figs. 5 and 7) with sandwiched (Figs. 6 and 8) sections. Sections were mounted on carbon-filmed grids and the sandwiched sections were in addition covered with a film of collodion.

FIG. 5. Unsandwiched section of rabbit bone marrow. Four granules (*G*) are present in the micrograph. The granule at the top contains holes with sharp edges which arise due to shrinkage accompanied perhaps by peeling away from the carbon substrate. There appears to be, at least on the right side of this granule, a cortical and a medullar region, but this is difficult to be certain of. Three other granules have rather indistinct and irregular edges and one of them (arrow) appears to be surrounded by a loose, membranous envelope. × 34,000.

FIG. 6. Sandwiched section of the same type of cell in rabbit bone marrow as is shown in Fig. 5. Three granules are present in this micrograph. All contain in the central regions a number of small, irregular particulates of low density which are completely missing from the granules of Fig. 5. The granule at the top possesses distinct cortical and medullar regions and in the original micrograph, at least, it is quite apparent that the cortical region is grainy in texture. The medullar region is partly of very low density. Whether this represents a distortion not eliminated by sandwiching is not known. The middle granule of the three is clearly enveloped in a membranous sack which is much more distinct than the corresponding feature thought to be present around a granule in Fig. 5. × 34,000.

FIG. 7. Unsandwiched section of mitochondria isolated from rat liver. The presence of sharp edged holes and the indistinctness of the outer limiting membranes of the mitochondria are consistent findings in sections of this material. × 48,000.

FIG. 8. Sandwiched section of mitochondria isolated from rat liver. The sharp edged holes present in the unsandwiched section are absent here, while the outer limiting membranes stand out with much greater distinctness. × 48,000.

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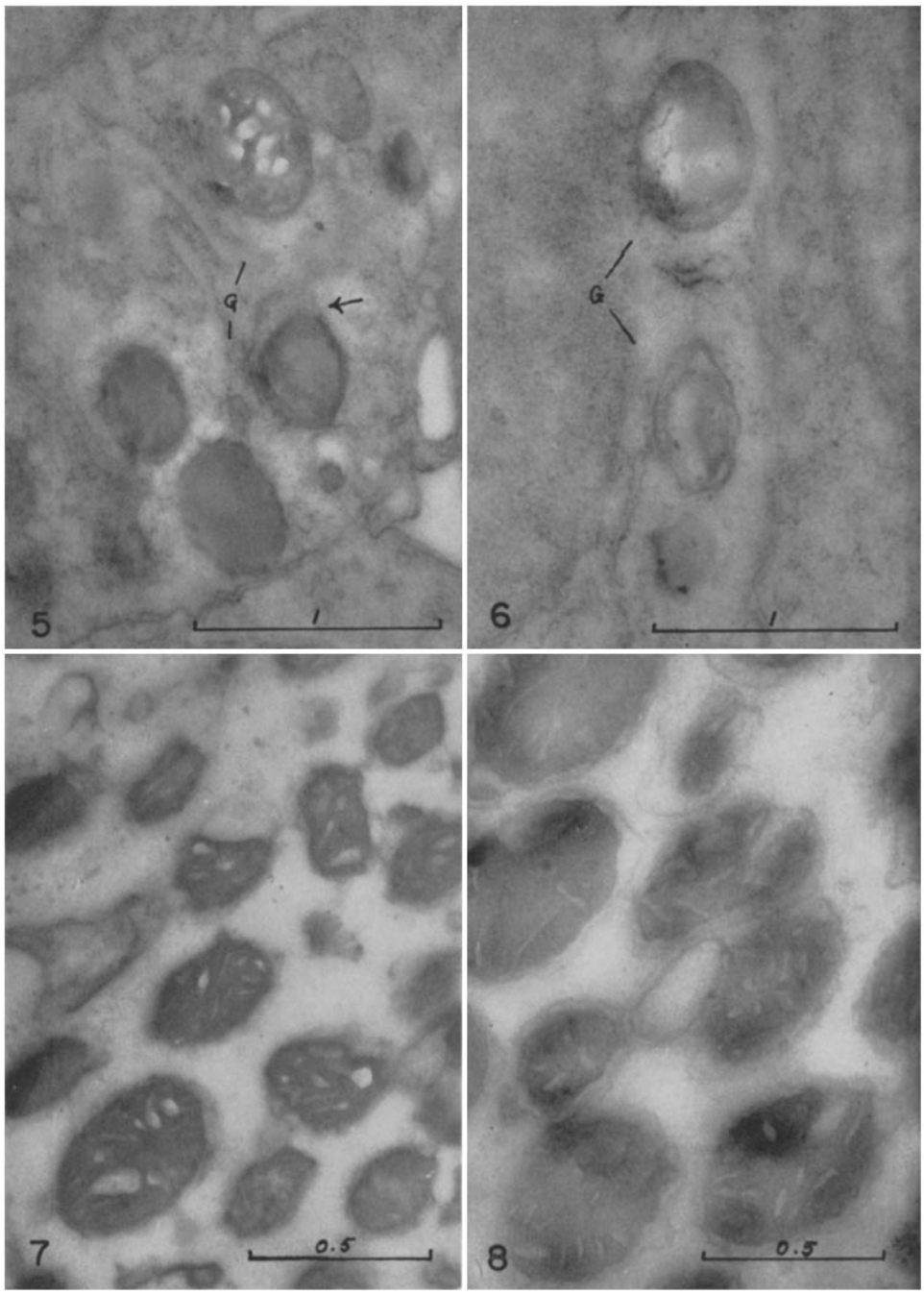

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PLATE 316

Comparison of sandwiched and unsandwiched sections of connective tissue of the leech in the vicinity of the gut. The section in Fig. 9 was supported on a carbon film, while that in Fig. 10 was mounted on collodion supported by a carbon net and was covered in turn by a second film of collodion.

FIG. 9. Unsandwiched section. Filaments of low density are sparsely scattered throughout the area, some of them lying in roughly parallel arrays in the plane of the section while others are sectioned perpendicularly. Two elements are present (*cp*) which are thought to be sections of cell processes. Because of the high contrast paper used to show the filaments these elements appear dense in the micrograph. × 28,000.

FIG. 10. Sandwiched section. An abundance of filaments of relatively high density were present in many areas of sandwiched sections of this material. In contrast to this, in the unsandwiched section (Fig. 9) it was difficult to find areas in which the filaments could easily be seen. An element of the type described above (*cp*) is also present in this micrograph. Instead of being of high density relative to that of the filaments, the density of this element is almost the same as the filament density. It is thought that the higher density and quantity of filaments in the sandwiched section indicates that material is lost from the unsandwiched section which is retained in the former. × 28,000.

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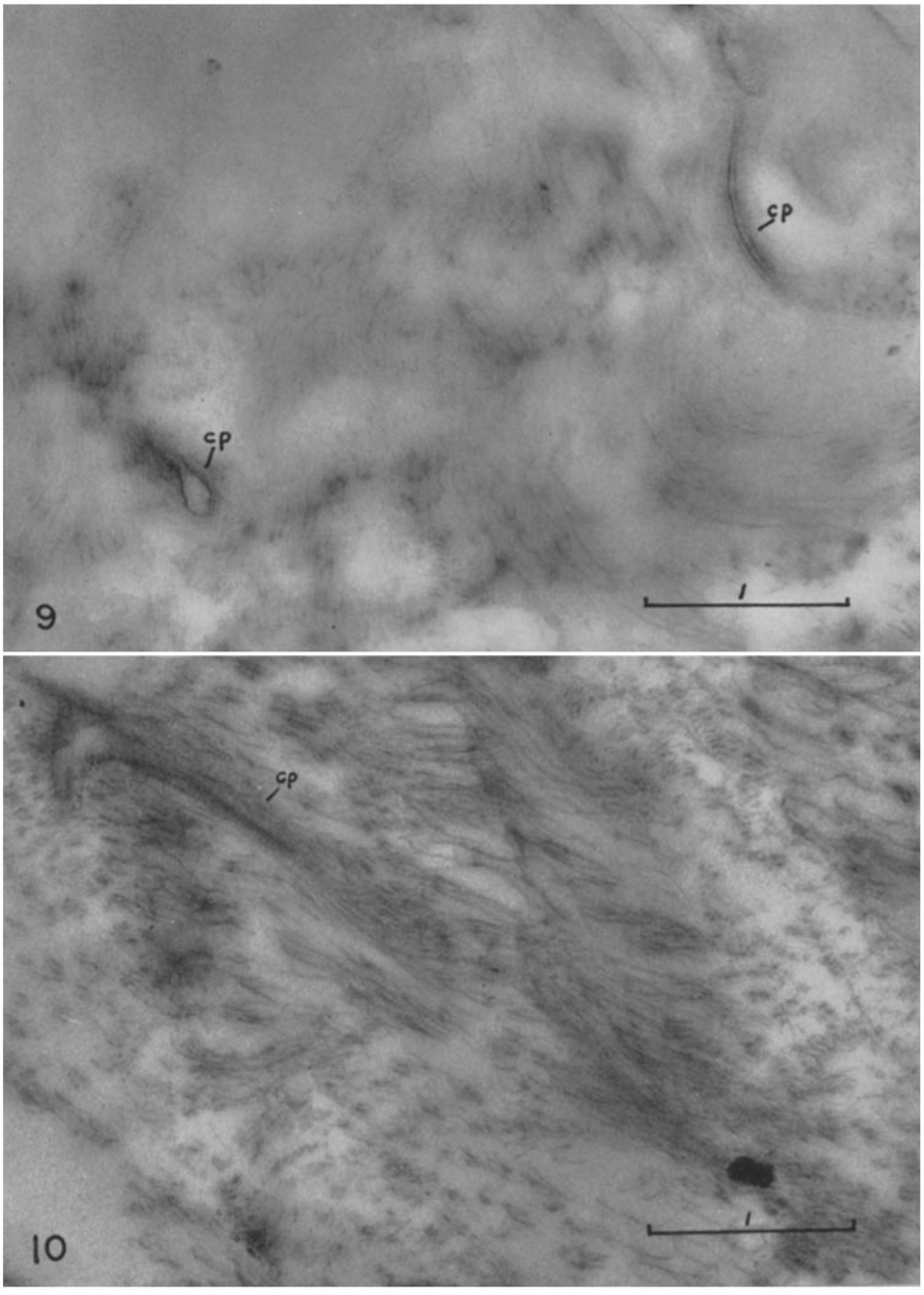

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PLATE 317

FIG. 11. Sandwiched section of the cell membrane of abdominal muscle of the leech. The section was mounted between collodion films and supported on a carbon net. The cell membrane extends roughly vertically in the center of the micrograph. The interior of the cell is on the right and the exterior on the left. The cell is coated with a thick, slightly granular layer of material of low density. Just within the cell wall are clustered numerous vesicular elements. To the right of these lie the muscle filaments. The cell wall and the walls of the vesicular elements are double in structure with a center-to-center spacing of 30 to 50 A which is clearly resolved at various points. × 132,000.

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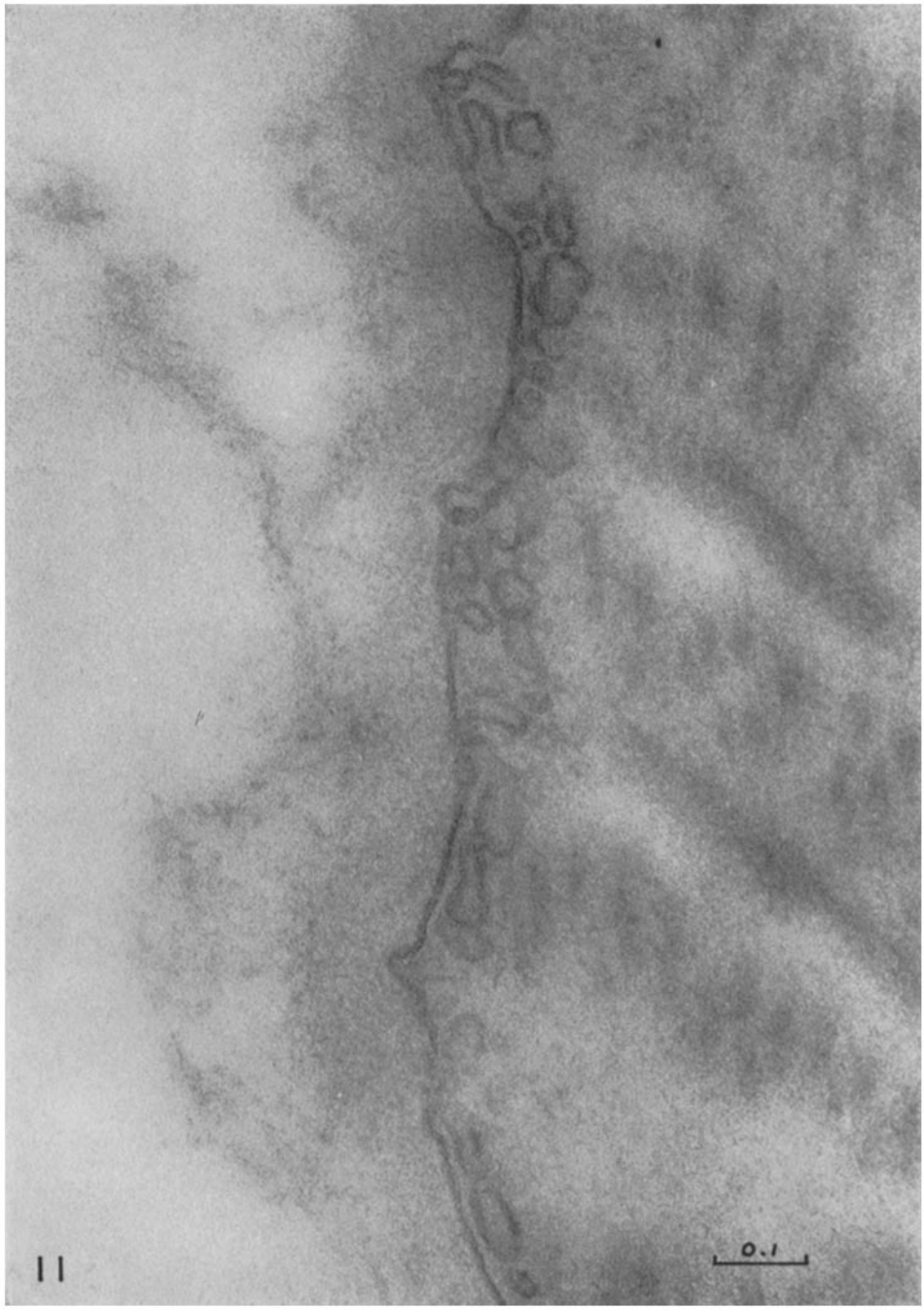

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