Staining of Tissue Sections for Electron Microscopy with Heavy Metals*

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PLATES 226 TO 229

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ABSTRACT

Heavy metals may be incorporated from solution into tissue sections for electron microscopy. The resulting increase in density of the tissue provides greatly enhanced contrast with minimal distortion. Relative densities of various structures are found to depend on the heavy metal ions present and on the conditions of staining. Certain hitherto unobserved details are revealed and some sort of specificity exists, although the factors involved are not yet understood.

At the present time low contrast is an important factor limiting the resolution which can be obtained with the electron microscope of small biological details in thin sections. As a result, there has been little advantage in achieving resolutions lower than 20 A in micrographs of most sectioned material. The contrast of such details is directly related to the difference between the mass per unit area of the tissue detail and that of the surrounding embedding material remaining after exposure to the electron beam. In order to increase contrast (for a given microscopical set-up), either the embedding material must be removed entirely or the density of the tissue must be increased. Removal of the embedding material introduces excessive distortion, earlier claims of ours notwithstanding (16). Heavy metals have long been used for increasing contrast of biological materials in the electron microscope (1), and a number of workers have successfully introduced heavy metal atoms into the tissue before embedding. This work has, however, been concerned with relatively resistant structures such as collagen (2), muscle (3, 4), or nerve myelin (5, 6). In addition to this, there have been more or less successful attempts to emphasize specifically certain components of the tissue by staining before embedding (7–9). There are many cellular constituents of possibly delicate structure which, without staining, defy analysis because of their low intrinsic contrast. Since staining before embedding could distort such structures, it follows that staining after embedding is preferable. Gibbons and Bradfield (17) have described the staining of electron microscope sections using solutions of lanthanum nitrate or osmium tetroxide, but do not appear to have appreciated the great increase in contrast provided by certain heavy metal solutions. At the time of writing this article, Swift (13) has reported specific staining of nucleic acids, proteins, and polysaccharides in sections treated with solutions containing various metallic salts and other reagents. The present communication deals with methods resembling these, but with the emphasis placed on substantially increasing the contrast of sectioned material with minimal distortion. In agreement with Swift, we find some indication of specificity for chemical composition.

Methods

Tissues were prepared by conventional methods after fixation in veronal-acetate buffer containing

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sucrose (15) and 1 per cent or 2 per cent OsO_4 at 0–5°C. for ½ to 2 hours, depending on the tissue. Butyl polymethacrylate was the embedding material.

Thin sections, exhibiting blue to gray zero-order interference colors, were collected on 100-mesh, carbon-coated, copper grids. The sections were stained by floating the grids face down on the surface of a solution containing heavy metal ions. In the case of phosphomolybdic acid solution which dissolves copper, we have had fair success using this technique although much of the grid is often dissolved at the end of the staining period. Unmounted sections floated on the phosphomolybdate solution have given better results as described in the section on Results.

Staining was carried out at room temperature. Grids bearing stained sections were washed very briefly in distilled water and blotted dry at once. They were then sandwiched (10) by covering with zero-order blue films of formvar. Solutions tested together with what appeared to be satisfactory concentrations and times of staining are listed below:

Uranyl acetate	saturated	1 to 2 hours.
Phosphotungstic acid	10 per cent	1 hour.
Phosphomolybdic acid	10 per cent	2 hours.
Ammonium molybdate	10 per cent	2 hours.
Sodium uranate	saturated	2 hours.

Differentiation of uranyl-acetate-stained sections was attempted by further treatment for 1 hour with Na citrate buffer adjusted to pH 5.5 at either 0.005 M or 0.01 M, or with Na acetate buffer at the same pH and molarities.

RESULTS

The increase in density of many structures after staining with uranyl acetate solution is such that the final print may be made with a photographic paper about two grades lower in contrast than would be the case with the unstained section. This is illustrated in Figs. 1 and 2. Such an increase in density of the tissue has been noted in a variety of tissues including rat and mouse hepatic cells, rat ameloblasts, odontoblasts, smooth muscle, chick muscle, and chick fibroblasts. Uranyl acetate is incorporated with little specificity into most of the components of tissue normally observed, and while contrast is very greatly increased, the general picture with certain exceptions is similar to that obtained without staining. Fine-scale specificity is, however, not excluded: certain reactive groups in the section may combine with uranyl[1], while others receive none at all. Thus, we may expect uranyl to combine with phosphate groups. That there are other possible sites of uranyl binding is indicated by the remarkable enhancement of collagen banding (Fig. 3), so that at least six bands can be easily seen in each 640 A period of fibers as small as 200 to 300 A in diameter. While collagen does not contain much phosphate, it is unique among proteins in its high periodic concentrations of polar groups which may be the sites of binding.

A number of differences are noted in sections of liver after staining with uranyl acetate. The mitochondrial matrix becomes much coarser in structure than is readily apparent in the unstained section (compare Figs. 1 and 2). This is probably not the result of clumping or distortion by the staining procedure, since a similar coarseness is sometimes faintly discernible without treatment; but it is probably the result of preferential staining of one component over another. The intramitochondrial granules do not take up uranyl appreciably, for, while still present, they are often difficult to detect. The membranes of the mitochondria stand out distinctly after staining and the membranes and contents of the endoplasmic reticulum and the associated ribonucleic acid (RNA)-containing particles are also stained.

Dense, heavily staining bodies 0.1 to 0.2 micron in diameter are frequently found scattered about the cytoplasm of hepatic cells in both rat and mouse. These are of two types: one containing very small, dense particles and described by Novikoff *et al.* (11) and by Richter (12), and the other, with no such particles. The latter type, without dense particles, appears to have escaped attention. It is characterized in the mouse by a single outer membrane enclosing a granular matrix resembling that of uranyl-stained mitochondria, but of higher density. Lying in this is an angular, folded, or laminated structure of higher density than the matrix and having a thickness of from 150 to 200 A (Figs. 4 *A* and *B*). Staining reveals that this structure contains ordered elements (Fig. 4 *B*).

An effort was made to improve the specificity of staining with uranyl-acetate. Sections treated with uranyl-acetate as described above were then extracted with a dilute solution of citrate or acetate. Since complexing of citrate or acetate with uranyl ions forms soluble compounds, it was thought that uranyl loosely bound in the section might be removed in preference to more tightly bound ions. Unfortunately, while uranyl was

[1]The word "uranyl" is used loosely here since it is only one of a number of uranium-containing ions present in solution at pH 4. Uranyl will be the predominant ion only at values of pH in the range of 1–2.

certainly removed, the procedure appeared to have little if any specificity.

Sections treated with molybdate (Fig. 5) and uranate were generally less dense than those treated with uranyl. Most noticeably stained with molybdate were the RNA-containing particles and certain cytoplasmic membranes, while these and the lysosomes particularly were stained with uranate. Membranes exposed to molybdate were sharper and thinner than when treated with either uranyl or uranate. Neither uranate nor molybdate brought out the coarser component of the mitochondrial matrix so noticeable with uranyl.

With phosphotungstic and phosphomolybdic acids, rather interesting results were obtained. Both reagents stained collagen heavily, although phosphotungstic acid appeared to be the more effective. Phosphotungstic acid produced a small and probably not useful generalized staining of most intracellular material, against which collagen stood out in striking relief (Fig. 6). In contrast to this, collagen in unstained sections is usually difficult to visualize (Fig. 7). No other tissue component was found to stain appreciably with phosphotungstic acid used in the manner described here. Phosphomolybdic acid, on the other hand, stained numerous small (200 to 1000 A in diameter) particles in liver which because of their size and distribution may represent deposits of glycogen (Fig. 8). These particles were of irregular shape and contained homogeneous, moderately dense material surrounded by a more heavily staining membrane or cortex (Fig. 9). They were never found within mitochondria or nuclei, always lay within the cell boundaries, and did not appear within cells of the liver other than the hepatic cells. The reaction was equally successful in a number of blocks of mouse and rat liver; however, it was undependable or impossible to elicit in blocks of tissue embedded more than 2 years previously. The heavy staining of the particles did not appear gradually as the staining time increased, but abruptly after 1½ to 2 hours' exposure to the staining solution. Incubation of sections in saliva before staining did not prove to be an adequate test for the hypothesis that the particles represented glycogen, since while the staining reaction was inhibited (Fig. 10), saliva inactivated by heat also inhibited the reaction. In those sections incubated with normal saliva, although densely staining particles were not found, "empty" vesicles corresponding in size and shape to the particles were present which may represent the more heavily staining cortex described above in the unincubated but stained sections (Fig. 10). More secure evidence is necessary before the nature of the staining particles can be demonstrated.

Staining with phosphomolybdic acid was found to present certain problems which deserve mention. Staining with this reagent was non-uniform throughout sections, some areas and even parts of cells receiving considerably more stain than others. Some of this non-uniformity appeared to be due to destaining during washing. Since the phosphomolybdate solution dissolves copper grids, it was found preferable to stain unmounted sections. To permit rapid washing, the sections were mounted on grids immediately after staining. Unmounted sections were picked up from the staining solution by lifting them from beneath in the usual way onto carbon-coated grids. Excess solution was blotted away and the grid allowed to dry. It was then washed by sweeping once through a small beaker of distilled water and blotted immediately by placing face down on facial tissue. No solution is trapped between the section and the carbon film in this procedure because the forces between the two films are sufficient to drive it out. Rapid washing does not, however, prevent all of the non-uniformity of staining observed, and this remains a problem to be dealt with.

Adjustment of pH of the staining solutions towards greater solubilities (*i.e.*, acidification of uranyl solution to pH 3 and alkalization of phosphotungstate solution to pH 4) reduced the intensity of staining. In particular, the intense staining of collagen by phosphotungstate and of liver particles by phosphomolybdate at pH 4 was much reduced.

Certain structures appear larger after staining than before. Most noticeable among these are the smallest collagen fibers and the network of material visible in the nucleus. It is believed that this is not an actual increase in size, but is the result of improved visibility of the edges of these structures. In support of this, it is noted that collagen banding revealed by staining is highly ordered all the way across the fiber, which might not be the case had the fiber swelled or if stain were precipitating onto itself on the outside of the fiber. Significantly, collagen and nuclear material are usually of low density in unstained sections.

DISCUSSION

High resolution staining of sections for the electron microscope can be accomplished ideally when the only effect upon the tissue structure is to increase its density at definite points. Other alterations in morphology will limit the resolution according to the dimensions of the alteration. In general, we can designate two types of stains for the electron microscope: those which are recognizable by virtue of topographical changes in tissue structure and those where a marked change in density is the primary effect. The limit of meaningful resolution achievable by density staining depends on the dimensions of the heavy metal groups deposited at each reactive site, upon the presence of possible polymerization reactions which may take place between closely lying groups, and upon the minimum dimensions of meaningful biological detail in the section. It is hoped that if meaningful detail in the range of 5 to 20 A exists in sections, density staining will reveal it.

Phosphotungstic acid staining of collagen and phosphomolybdic acid staining of granules in the liver indicate the possibility of staining reactions with some degree of specificity. The present work describes techniques for enhancing contrast in sections so that details formerly masked by low contrast become visible and so that higher microscopical resolutions may become useful in revealing structure. The methods described are applicable to sections regardless of the method of fixation and probably do not introduce additional topographical distortions above the 10 A level. These techniques may well be specific for a limited number of particular reactive groups in the fixed tissue. If this is true, a submolecular staining should be possible. The advantages of staining after sectioning are as follows: (1) there is less chance for distortion than when the tissue is stained before embedding; (2) serial sections of the same block of tissue can be compared following different treatments; (3) the staining is more uniform than it would be in tissue stained before embedding.

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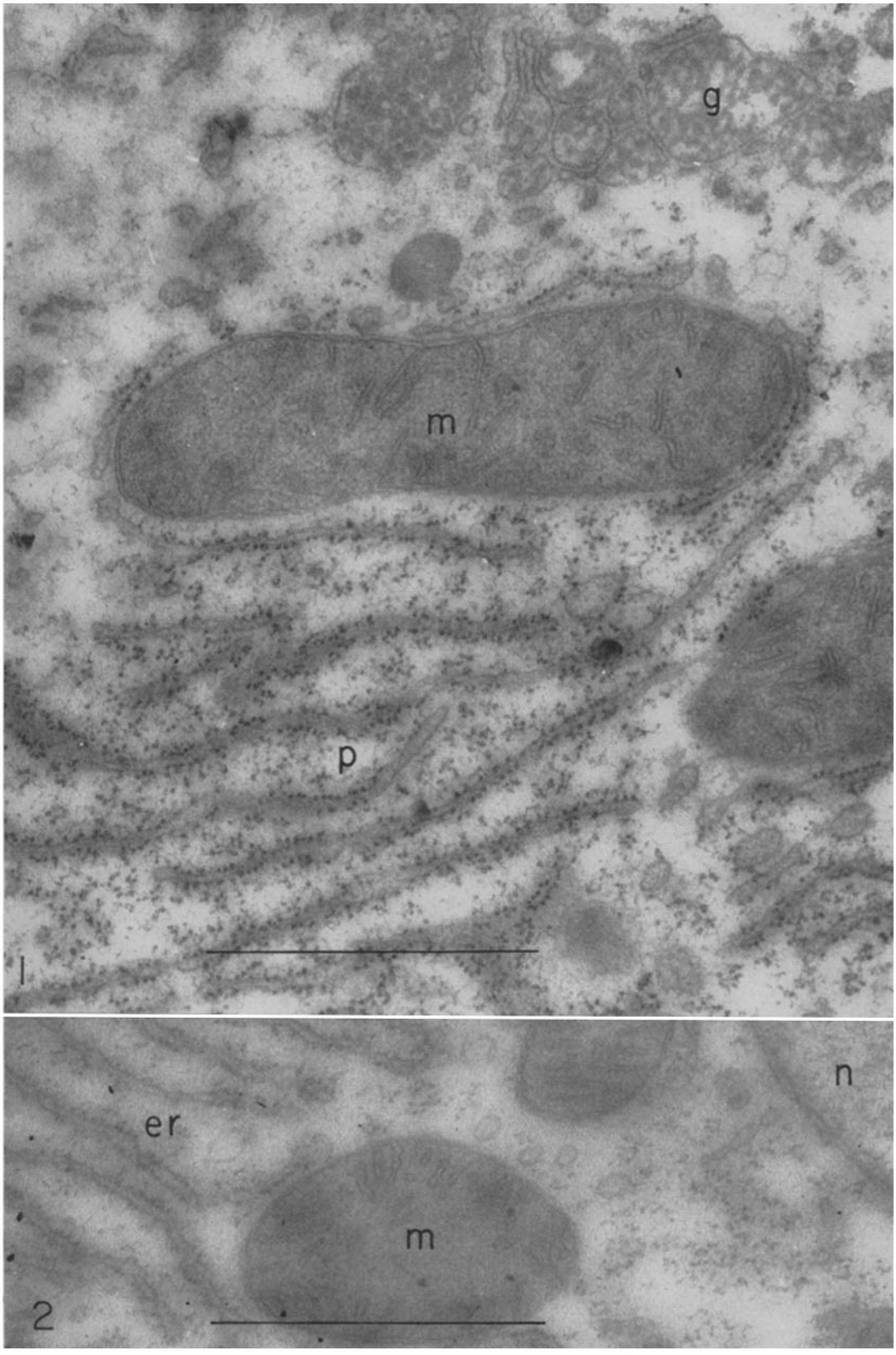
EXPLANATION OF PLATES

PLATE 226

FIG. 1. Micrograph of a sandwiched section of mouse liver stained for 2 hours in a saturated solution of uranyl acetate. The general appearance resembles that of unstained sections of the same material, except for greater contrast. The notable differences are the granular matrix of mitochondria (*m*) and the highly dense RNA particles (*p*). The Golgi region (*g*) shows the usual spherical and tubular vesicles and is characterized here by the unusually distinct membranes and the rather dense contents. Both of these features are primarily due to sandwiching, neither the membranes nor the contents being so easily discernible in unsandwiched sections. RNA particles stain intensely and membranes and contents of the endoplasmic reticulum to a lesser extent than the RNA particles. × 53,000.

FIG. 2. Micrograph of a sandwiched, but unstained section of mouse liver for comparison with Fig. 1. Both micrographs were made of sections of about the same thickness and from the same block of tissue. The image densities of the two negatives were about the same, and the final prints were made on photographic paper of the same grade of contrast. The generally lower contrast of the unstained section is obvious. Other differences include the smoothness of the mitochondrial (*m*) matrix and the lower density of the RNA particles relative to the membranes of the endoplasmic reticulum (*er*). The envelope of the nucleus (*n*) is also much less dense. × 53,000.



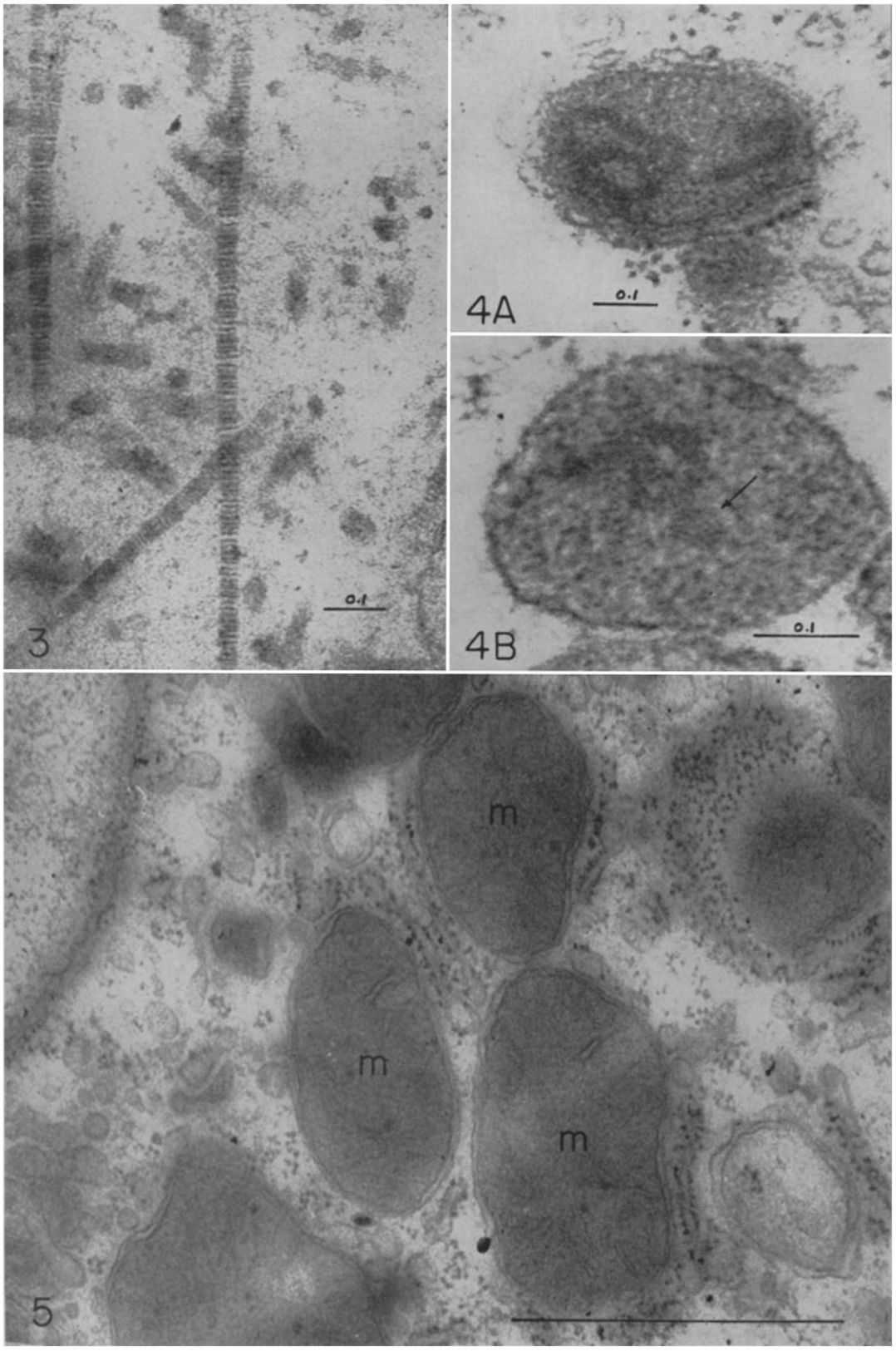
(Watson: Staining of tissue sections)

PLATE 227

FIG. 3. Micrograph of a sandwiched section of rat incisal predentine after staining 2 hours in a saturated solution of uranyl acetate. Six subperiod bands are revealed in every major 640 A band. × 100,000.

FIGS. 4 *A* and *B*. Micrographs of sandwiched sections of mouse liver after staining 2 hours in a saturated solution of uranyl acetate, showing details of dense cytoplasmic bodies. The bodies are 0.1 to 0.2 micron in diameter and are enclosed by a single membrane. The contents are of two parts: a granular matrix resembling that of mitochondria, but of higher density and containing a folded and sometimes lamellar structure of higher density. The micrographs are consistent with the idea that this structure represents a folded sheet about 150 A in thickness. Figure 4 *A* shows the internal sheet in profile and Fig. 4 *B*, a portion of it oriented tangentially. In the tangential view a small-scale order can be discerned (arrow). *A*, × 100,000. *B*, × 170,000.

FIG. 5. Micrograph of a sandwiched section of mouse liver after staining 2 hours in a solution containing 10 per cent ammonium molybdate. Staining with molybdate is generally less intense than with uranyl, with the exception of RNA particles and certain membranes including those of mitochondria. Molybdate-stained membranes are thinner and more distinct than in the case of uranyl. The mitochondrial (*m*) matrix, although darkened somewhat, does not appear granular with molybdate. × 53,000.

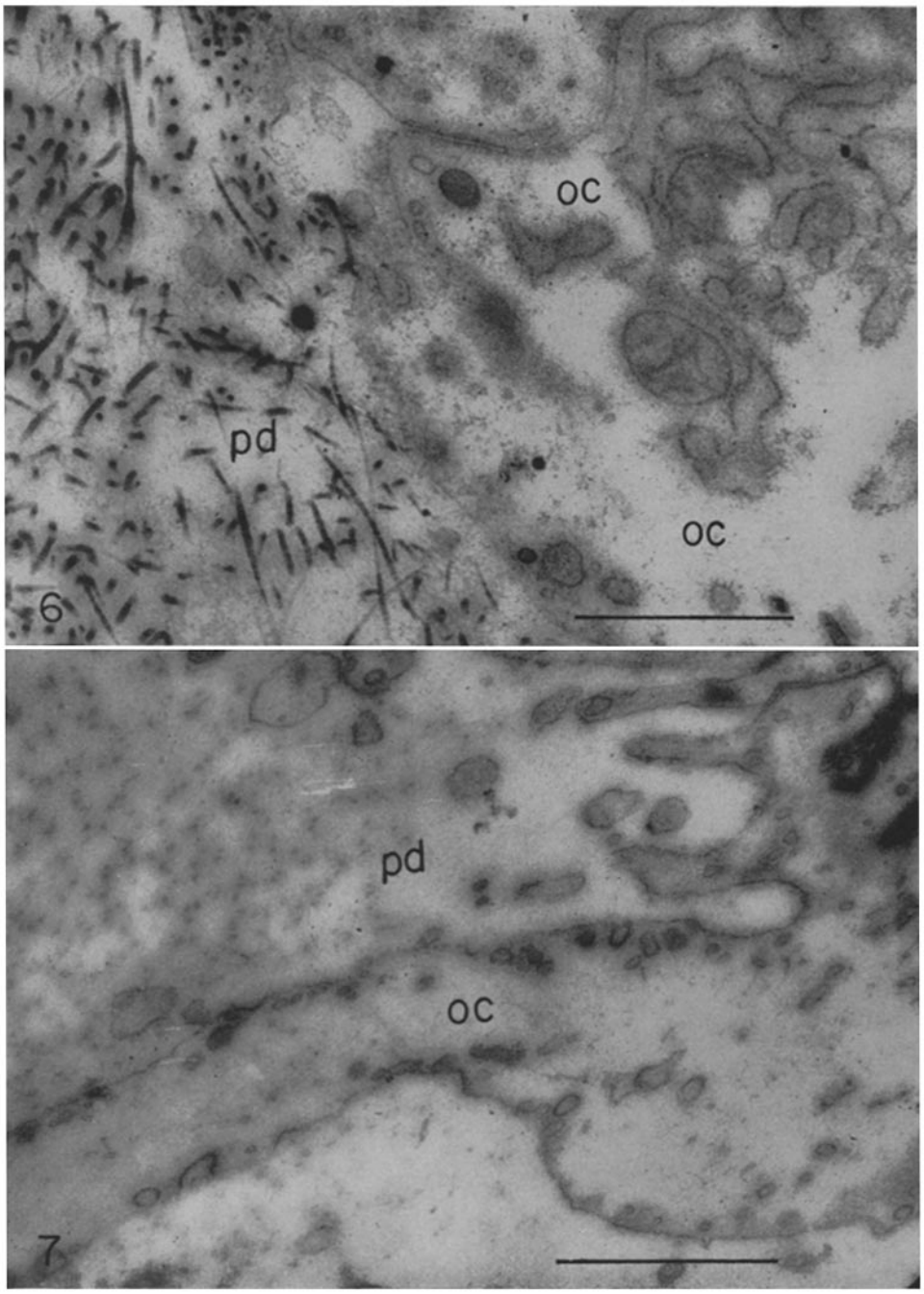


(Watson: Staining of tissue sections)

PLATE 228

FIG. 6. Micrograph of a sandwiched section, showing the border between predentine and ondontoblasts in the lower incisor of the rat after staining for 1 hour in a solution containing 10 per cent phosphotungstic acid. Predentine (*pd*) is on the left, and the cytoplasm of odontoblasts (*oc*) on the right of the micrograph. The extremely high density of the small (200 to 330 A in diameter) collagen fibers relative to that of the cytoplasm of the ondontoblasts is characteristic of PTA staining and has been observed in all collagenous tissues examined. × 30,000.

FIG. 7. Micrograph of a sandwiched but unstained section of the border between predentine (*pd*) and ondontoblasts (*oc*) in the lower incisor of the rat. Collagen fibers are of such low density that in this relatively thick (*ca.* 800 A) section only those fibers oriented perpendicular to the section are visible. Cytoplasmic structures of the odontoblasts are of much higher density than the collagen in contrast to the situation where PTA is used (Fig. 6). × 30,000.



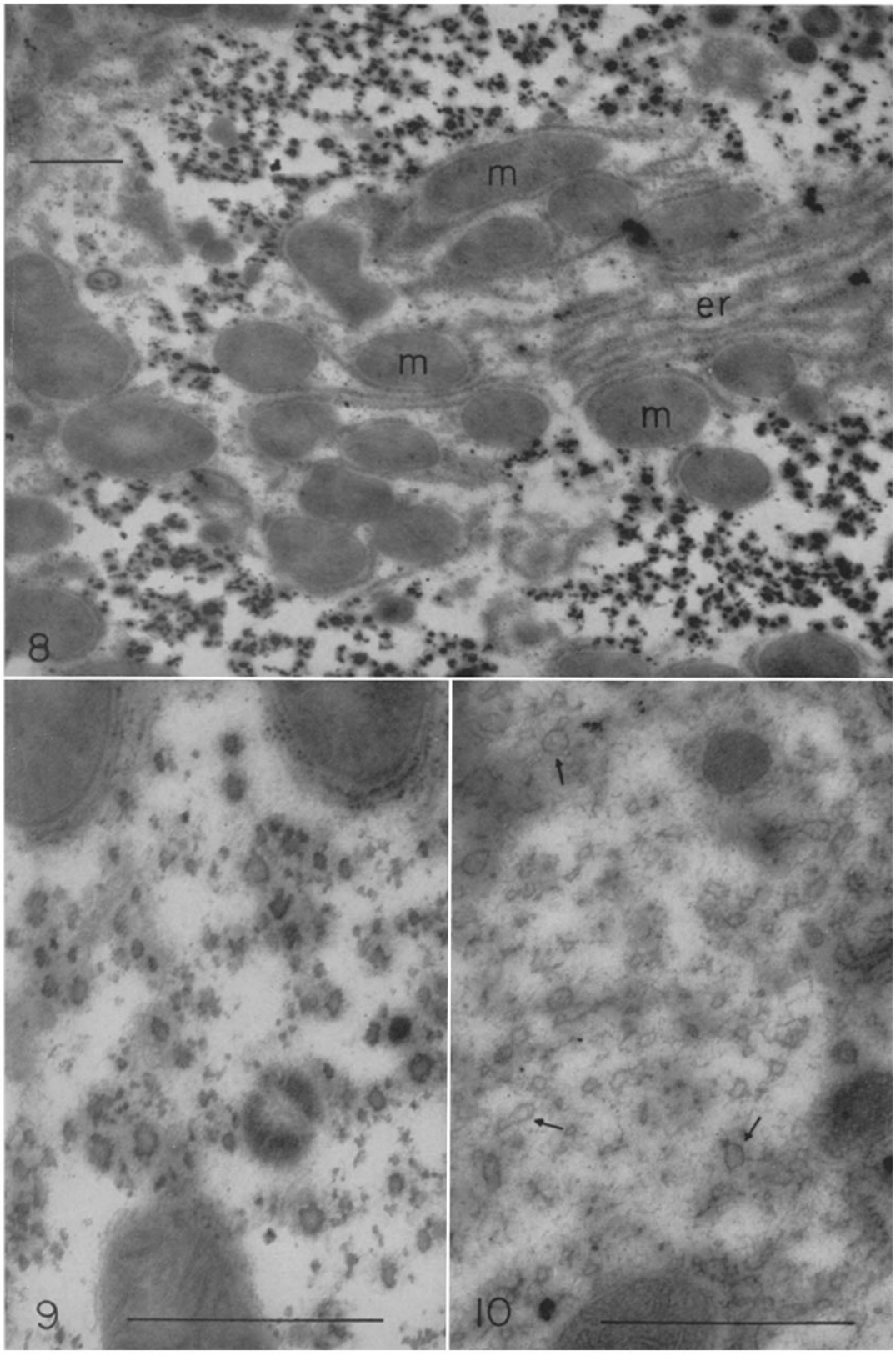
(Watson: Staining of tissue sections)

PLATE 229

FIG. 8. Micrograph of a sandwiched section of mouse liver after staining for 2 hours in a solution containing 10 per cent phosphomolybdic acid. Mitochondria (*m*) and endoplasmic reticulum (*er*) occupy the center of the micrograph and are surrounded by regions containing numerous, densely stained particles which are thought to represent deposits of glycogen. Three or four dense particles at the upper right in the region of endoplasmic reticulum and mitochondria are contamination. Phosphomolybdate-stained particles are never found within mitochondria, endoplasmic reticulum, or nuclei. × 15,000.

FIG. 9. Micrograph of an area similar to that in Fig. 8 at higher magnification. The staining reaction was less intense in this region, so that details are easier to make out. The particles range in size from about 200 to 1000 A. They exhibit an intensely staining cortex or membrane surrounding a less dense central portion. × 40,000.

FIG. 10. Micrograph of a sandwiched section of mouse liver after incubation for 2 hours at 37°C. in saliva and followed by staining for 2 hours in phosphomolybdic acid. Although the intense reaction illustrated in Figs. 8 and 9 could not be found in sections first treated with saliva, this did not constitute a test for glycogen because a similar effect was produced by saliva first inactivated by boiling. The micrograph is of interest because in regions of cytoplasm not occupied by mitochondria or endoplasmic reticulum, numerous vesicles (arrows) were often present having the same general dimensions as the particles intensely stained by phosphomolybdate. × 40,000.



(Watson: Staining of tissue sections)