Staining of Tissue Sections for Electron Microscopy with Heavy Metals

II. Application of Solutions Containing Lead and Barium*

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(Received for publication, August 1, 1958)

ABSTRACT

Descriptions of three heavy metal stains and methods of application to tissue sections for electron microscopy are presented. Lead hydroxide stains rather selectively two types of particles in liver: those associated with the endoplasmic reticulum and containing ribonucleic acid and other somewhat larger particles. Barium hydroxide emphasizes certain bodies within vesicles of the Golgi region of hepatic cells. Alkalized lead acetate is useful as a general stain, as are also lead and barium hydroxides.

The use of solutions containing heavy metals as stains for sections of tissue for the electron microscope has been described earlier (1). In an extension of this study the application of barium- and lead-containing solutions is presented here.

Methods

Small pieces of rat or mouse liver were fixed for 1½ hours at 0–5 degrees C. in 1 per cent OsO_4 buffered to pH 7.3 with veronal-acetate and containing sucrose as described by Caulfield (2). They were embedded after dehydration in *n*-butyl methacrylate. Sections exhibiting silver interference colors after flattening in xylol vapor (3) were collected on carbon-coated grids. Staining was carried out by floating the grids, section side down, on the surface of a staining solution for periods of from 15 seconds to 2 hours. Stained sections were rinsed by passing the grid two or three times rapidly through distilled water contained in a beaker and immediately blotted with filter paper. All sections were sandwiched by covering with a thin film of formvar (4).

Lead Acetate.—A saturated solution of lead acetate is used. This will stain without further preparation, but more intense stain results if the solution is alkalized. To 2 ml. of solution in a small beaker, 40 per cent NaOH is added drop-wise. The precipitate formed is dissolved by stirring after each addition. The solution is ready for use when a faint opalescence forms which is permanent on stirring. This solution or suspension is not stable and should be used at once.

Barium Hydroxide.—A saturated solution is used without adjustment of pH. Insoluble barium carbonate is readily formed on contact with the air and causes the solution to become cloudy. It will clear on standing in a closed container and some of the clear solution may then be withdrawn for use with a pipette. Before use, the surface of the solution should be cleaned by drawing a filter paper across it and the grid then immediately floated. It is difficult to prevent the formation of a film of barium carbonate on the surface unless the staining is carried out in a CO_2-free atmosphere; however, this film can be floated from the grid following staining by brief rinsing in distilled water.

Lead Hydroxide.—A nearly saturated solution of lead acetate, $Pb(C_2H_3O_2)_2 \cdot 3H_2O$, is prepared by dissolving 8.26 grams of the lead salt in 15 ml. of distilled water. This can be accomplished rapidly by grinding the lead salt in the water with a glass rod. Into this is blown rapidly from a pipette, 3.2 ml. of aqueous 40 per cent NaOH. Stir vigorously for a moment and centrifuge briefly to sediment the precipitate. Determine the volume of supernatant, discard, and resuspend the precipitate in an equal volume of distilled water. Centrifuge, discard the supernatant, and resuspend again in an equal amount of distilled water. Centrifuge a third time. The resulting super-

* These investigations were performed in part under contract with the United States Atomic Energy Commission at the University of Rochester, Atomic Energy Project, Rochester, New York, and in part with funds provided by Research Grant CY-3589 (C1) from the National Institutes of Health, United States Public Health Service.

natant is the staining solution. This may be stored in a bottle provided with a dropper. A small quantity of precipitate should be included with it to maintain saturation. The staining solution appears to keep well, but should be stored at room temperature and *not* in the cold. This method of preparation results in solutions which appear to be uniform in their staining properties. Lead forms a number of different hydroxides and it is possible by other procedures to prepare solutions of lead hydroxide which do not uniformly stain certain particles in liver cells. Staining is carried out for 1 to 45 minutes, depending on the purpose (see text). The grid is then rinsed in distilled water and blotted dry.

RESULTS

All components of cells and tissues including membranes, contents of nuclei, particles containing ribonucleic acid (RNA), miofibrils, collagen, etc., are stained with lead acetate. The present detailed discussion will, however, be confined to liver. In general, most intense staining results when the section is exposed for short periods, *i.e.*, less than 5 minutes, to the staining solution. Membranes become particularly distinct after lead acetate treatment (Fig. 1), while lipide granules and matrices of various cell organelles are less dense. RNA-containing particles are often somewhat emphasized and, while the contents of nuclei are stained, there is little obvious selectivity of one component over another. With periods of staining longer than 10 minutes, ferritin (6) particles begin to disappear.

Sections exposed to barium solutions for less than 1 minute resemble those stained by uranyl (1) although there is less contrast, in part, probably, due to the lower atomic number of barium. As the staining time is increased, one component, the rather diffuse, globular elements within vesicles of the Golgi zone of hepatic cells (9), increases greatly in density so that this material stands out markedly in the section (Fig. 2). Barium also stains the periphery of lipide inclusion bodies intensely. The optimum time of treatment with barium hydroxide is about 5 minutes. As the exposure time increases beyond this, the homogeneity of the cytoplasmic matrix is reduced and a fine network results, indicating extraction and possibly reorganization of matrix fine structure. This is not surprising in view of the high alkalinity, *ca.* pH 13.5, of the solution used.

Lead hydroxide presents a picture rather different from lead acetate. It is a very vigorous stain, particularly for certain irregularly shaped particles in the hepatic cells (Fig. 3). These are probably the same as particles stained by phosphomolybdic acid which have been suggested as representing deposits of glycogen (1, 5). Lead hydroxide as a stain for these particles has the great advantage over phosphomolybdic acid that the staining appears to be uniform over the entire section, all particles present being intensely stained, not only in a given section, but in sections from different blocks of liver. Lead hydroxide also stains intensely, but rather less uniformly, the RNA-containing particles (Fig. 4). These observations apply particularly for short staining times of 1 or 2 minutes. When sections are exposed for 30 to 40 minutes to the lead solution, more general, very intense staining results (Fig. 5).

DISCUSSION

Two of the three heavy metal stains described here, lead and barium hydroxide, exhibit marked affinity for certain cytoplasmic structures. Since the nature of this affinity and the chemical nature of the material preserved in sections is unknown, interpretation of the specificities described and correlation of results in a variety of tissues will have to be treated with caution. Lead hydroxide stains strongly RNA-containing particles in a variety of cells. Whether it has, in this case, an affinity for RNA, ribonucleoprotein or a particular protein is not known. It also stains somewhat larger particles in liver and in embryonic heart muscle, but we have not found such particles in diaphragm of adult rats.

Much of the contrast in electron microscope images arises from variations in mass of the specimen from one point to another. Such variations may be intrinsic in the specimen or, as pointed out by Ornstein (10), may arise due to differential losses of parts of the specimen on exposure to the electron beam. Some of the effects of heavy metal staining are probably due to differential extraction of tissue components during staining and to stabilization of certain components so that they are more resistant to the electron beam than before staining. The very marked increase in density of stained tissue sections, however, suggests that many components have undergone an appreciable increase in mass due to incorporation of heavy metal ions. That such increase in mass can occur in biological materials has been shown from densitometric measurements of stained viruses reported by Hall (11).

The effect of length of staining time is not, in

our experience, immediately predictable, but appears to be a complex function of the rate of adsorption of heavy metal ions, of extraction of tissue components from the section, and of alterations which may occur in the staining solution. The alkalized lead acetate solution probably contains lead hydroxide as well as lead acetate and is unstable in the sense that it loses its colloidal nature and forms a precipitate. It stains most effectively in a few minutes. Barium hydroxide also stains most heavily in a few minutes, due apparently to extraction of tissue components with long exposure of the section. Lead hydroxide exhibits greater "specificity" for the particles discussed earlier when applied for short periods, other structures increasing in density as the staining time is increased.

Probably it will turn out that lead hydroxide is the most generally useful of all the heavy metal stains we have described because of its affinity for many cellular structures and its high density. However, the other stains may reveal unexpected virtues when applied to tissues not reported here and, therefore, merit some attention.

Note Added in Proof.—Lead hydroxide and lead carbonate dry from solution to form extremely finely divided solids which redissolve only with difficulty. Since lead is toxic, care should be taken to avoid inhaling the dried salts or getting them on the hands.

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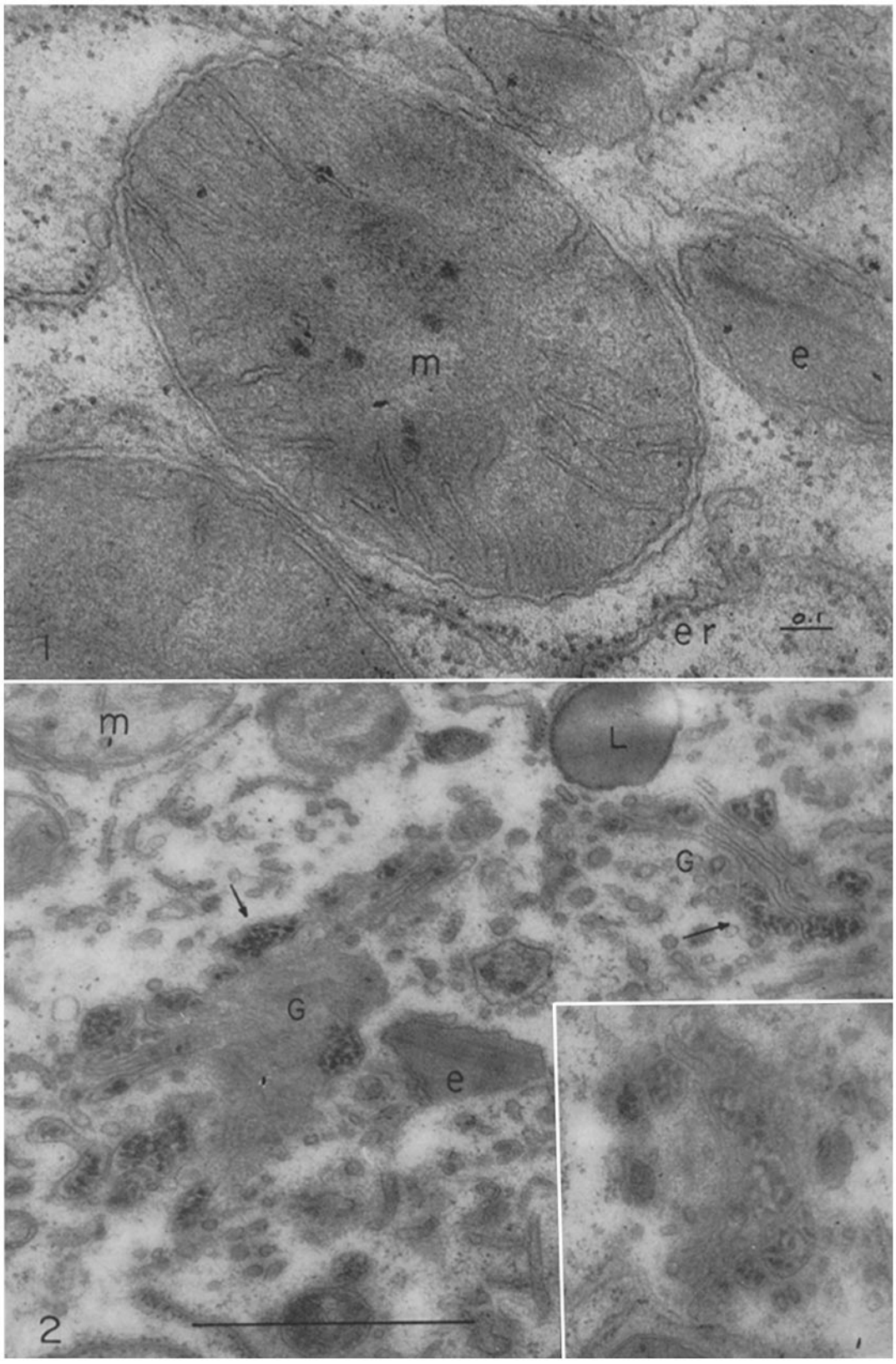
Explanation of Plates

Plate 361

Fig. 1. Electron micrograph of section of mouse liver after staining 5 minutes with a saturated solution of lead acetate. Membranes of mitochondria (*m*), microbodies[1] (*e*), and endoplasmic reticulum (*er*), stand out distinctly. The RNA-containing particles and the internal structure of microbodies are also stained while the matrices of mitochondria and microbodies are not as intensely stained as, for example, with uranyl. × 81,000.

Fig. 2. Electron micrograph of section of mouse liver after staining for 5 minutes with a saturated solution of barium hydroxide. This stain is particularly interesting because of the intense deposition of barium in globular masses of material within vesicles (arrows) of the Golgi regions (*G*) of liver. Barium hydroxide also stains rather heavily the cortex of lipide inclusions (*L*). RNA-containing particles show little, if any, increase in density; however, other organelles, mitochondria (*m*), microbodies (*e*), and membranes of the endoplasmic reticulum are well emphasized. Inset shows for comparison the appearance of the Golgi region in an unstained section of mouse liver. × 44,000.

[1] "Microbody" is a term originally applied by Rhodin (7) to bodies he observed in the kidney epithelial cells of the proximal convoluted tubules and, later, by Rouiller and Bernhard (8), to similar appearing bodies in hepatic cells.



(Watson: Staining with heavy metals. II)

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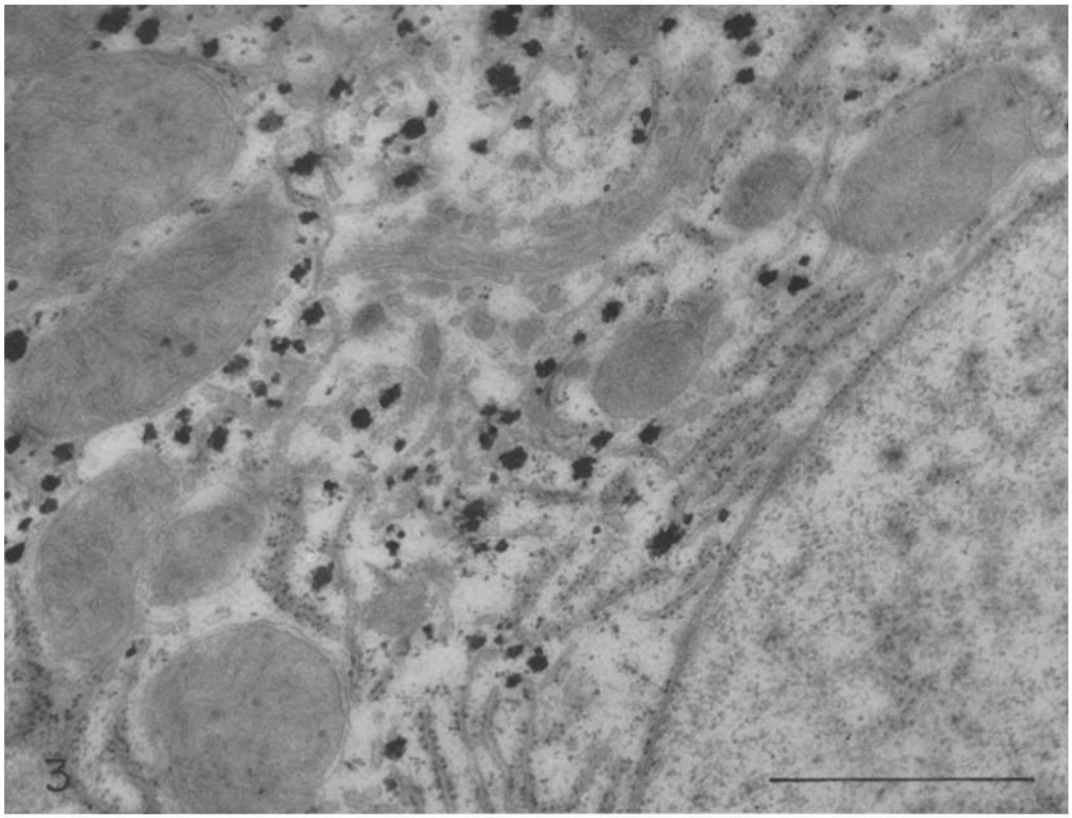
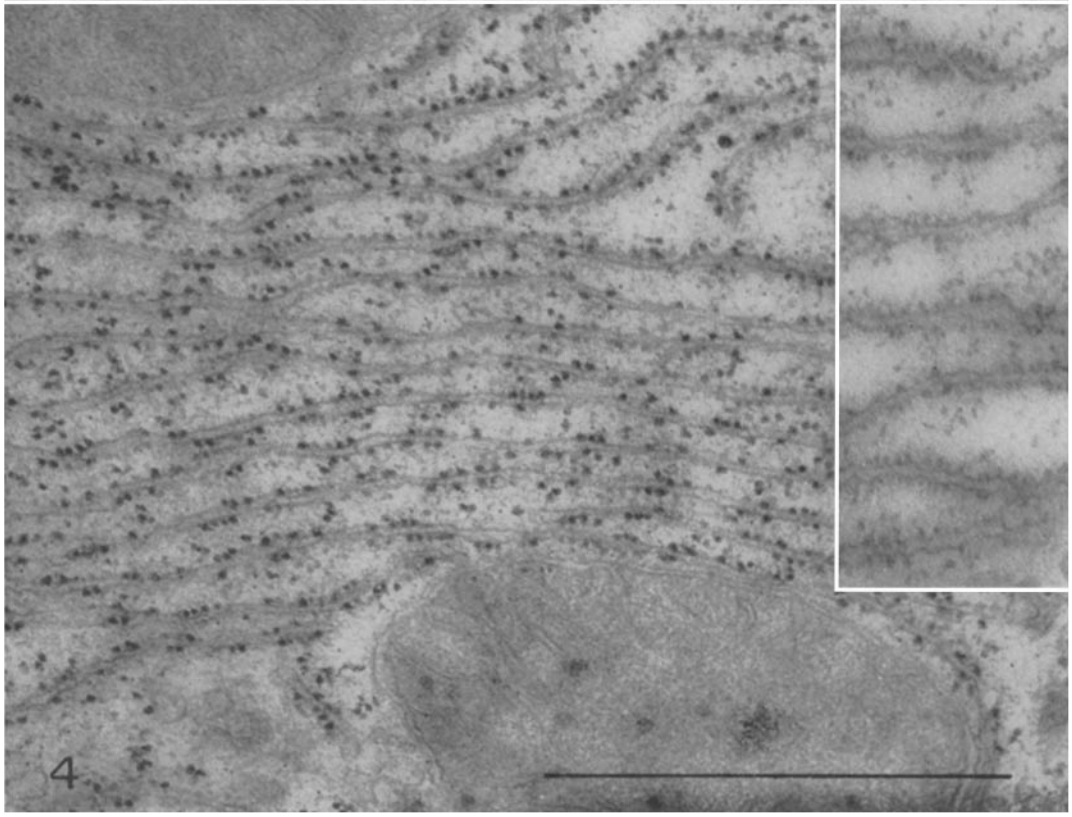
FIG. 3. Electron micrograph of section of rat liver after staining 2 minutes with a saturated solution of lead hydroxide. Irregularly shaped particles in the cytoplasm of the hepatic cells are very strongly stained. These particles, found outside of all other cell organelles, are probably the same as similarly shaped and situated particles stained with phosphomolybdic acid (1, 5) and may represent deposits of glycogen. × 35,000.

FIG. 4. Electron micrograph of a section of rat liver after staining 2 minutes with a saturated solution of lead hydroxide. The RNA-containing particles associated with the endoplasmic reticulum are markedly increased in density by short exposure to lead hydroxide solution. Inset shows for comparison the appearance of endoplasmic recticulum in an unstained section of liver. The RNA-containing particles appear larger after staining than before, but this is thought to be more apparent than real and due to the fact that the edges of particles are not readily discernible without staining. × 62,000.





(Watson: Staining with heavy metals. II)

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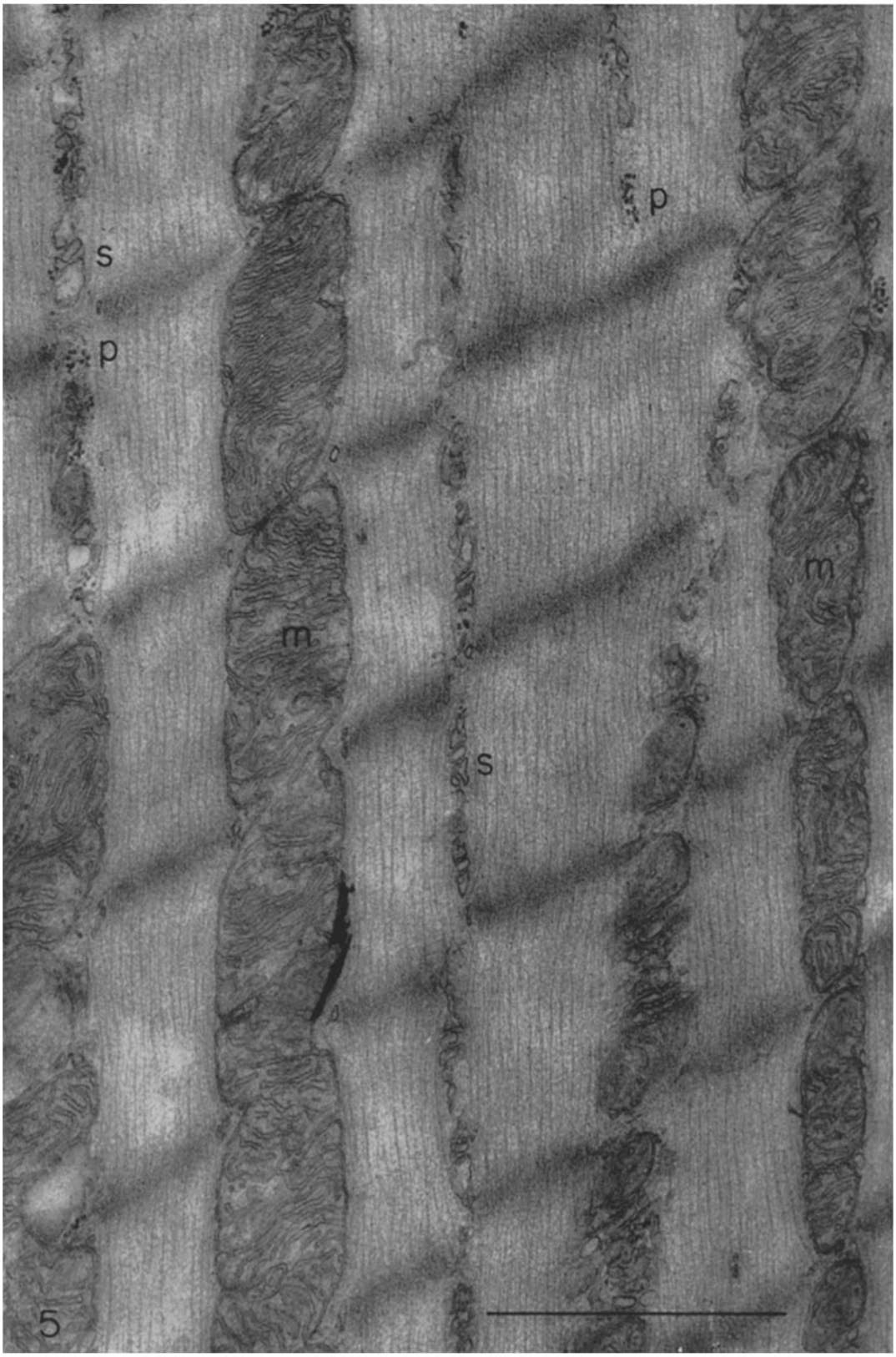
FIG. 5. Electron micrograph of section of rat diaphragm after staining 45 minutes with a saturated solution of lead hydroxide. General contrast in muscle is greatly increased, particularly in membranes of mitochondria (*m*) and sarcoplasmic reticulum (*s*) and in the myofilaments. Small particles (*p*), present in muscle, are particularly heavily stained. A dense needle-shaped mass closely applied to one of the mitochondria probably is contamination and has no biological significance. × 46,000.



(Watson: Staining with heavy metals. II)