SILVER IMPREGNATION OF ULTRATHIN SECTIONS FOR ELECTRON MICROSCOPY

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ABSTRACT

A new procedure is described for silver impregnation of thin sections for electron microscopy. Sections of various tissues, fixed in OsO_4 and embedded in methacrylate, were treated with an ammoniacal silver solution, directly or after oxidation with periodic acid or hydrogen peroxide. After OsO_4 fixation all cellular membranous systems exhibit a primary argentaffinity probably due to the reduction of ammoniacal silver solution by the reduced osmium bound to unsaturated lipids. Bleaching the sections with hydrogen peroxide removes the argentaffinity of protoplasmic structures. Treatment of the sections with periodic acid results in decreased argentaffinity of protoplasmic components while the argentaffinity of metaplasmic structures is greatly enhanced. The latter procedure appears particularly useful for enhancing the contrast of basement membranes.

Impregnation by heavy metals of thin sections of biological materials already fixed with OsO_4 may serve either to increase the contrast of particular structures or substances possessing low intrinsic or artificially induced density, or to study cytochemical localizations at the fine structural level.

Special procedures for staining with phosphotungstic acid (1–7), phosphomolybdic acid (7), and uranyl acetate (6, 7) have already been described in electron microscopy. In most cases, tissue specimens have been "stained" with heavy metals after OsO_4-fixation and before embedding. Watson has suggested that staining be carried out after the sections have been mounted on copper grids (8), but this procedure can not be used for silver staining because coarse precipitates form upon immersion of the copper grids in ammoniacal silver solutions.

A number of more or less successful attempts to adapt silver impregnation for electron microscopy have been, however, reported. As a rule, the impregnation is performed on tissue blocks after fixation in formaldehyde or OsO_4, that is, before the material is embedded, but with this technique the silver impregnation obtained is less selective and not homogeneous.

In my earlier attempts (12–15), I adapted Gomori's periodic acid–silver-methenamine stain, as modified by Jones (16) for both optical and electron microscopy but the method was rather complicated and lead to the loss of many sections.

The present report describes a new procedure for effective silver impregnation. The method is expeditive, gives satisfactory results in silver impregnation, and can be extended to other staining procedures which cannot be applied to materials mounted on copper grids.

MATERIALS AND METHODS

Small pieces of various animal tissues, fixed for 2 hours in 2 per cent OsO_4 buffered to pH 7.3 (following Palade), were dehydrated through a series of graded ethanols and embedded in a mixture of 1 part methyl- to 20 parts *n* butyl-methacrylate. Some pieces were treated for 3 hours with xylol before embedding. Sections exhibiting silver interference colors were transferred to a Petri dish containing distilled water

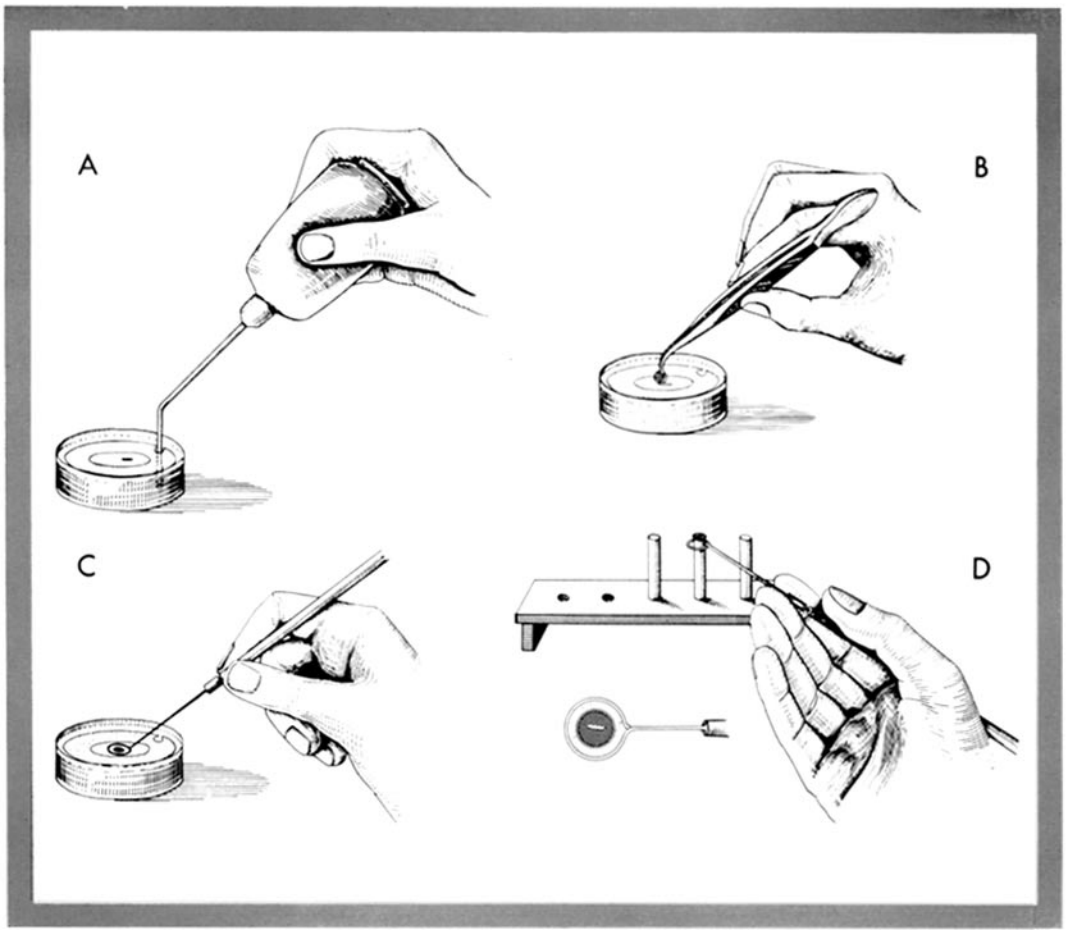
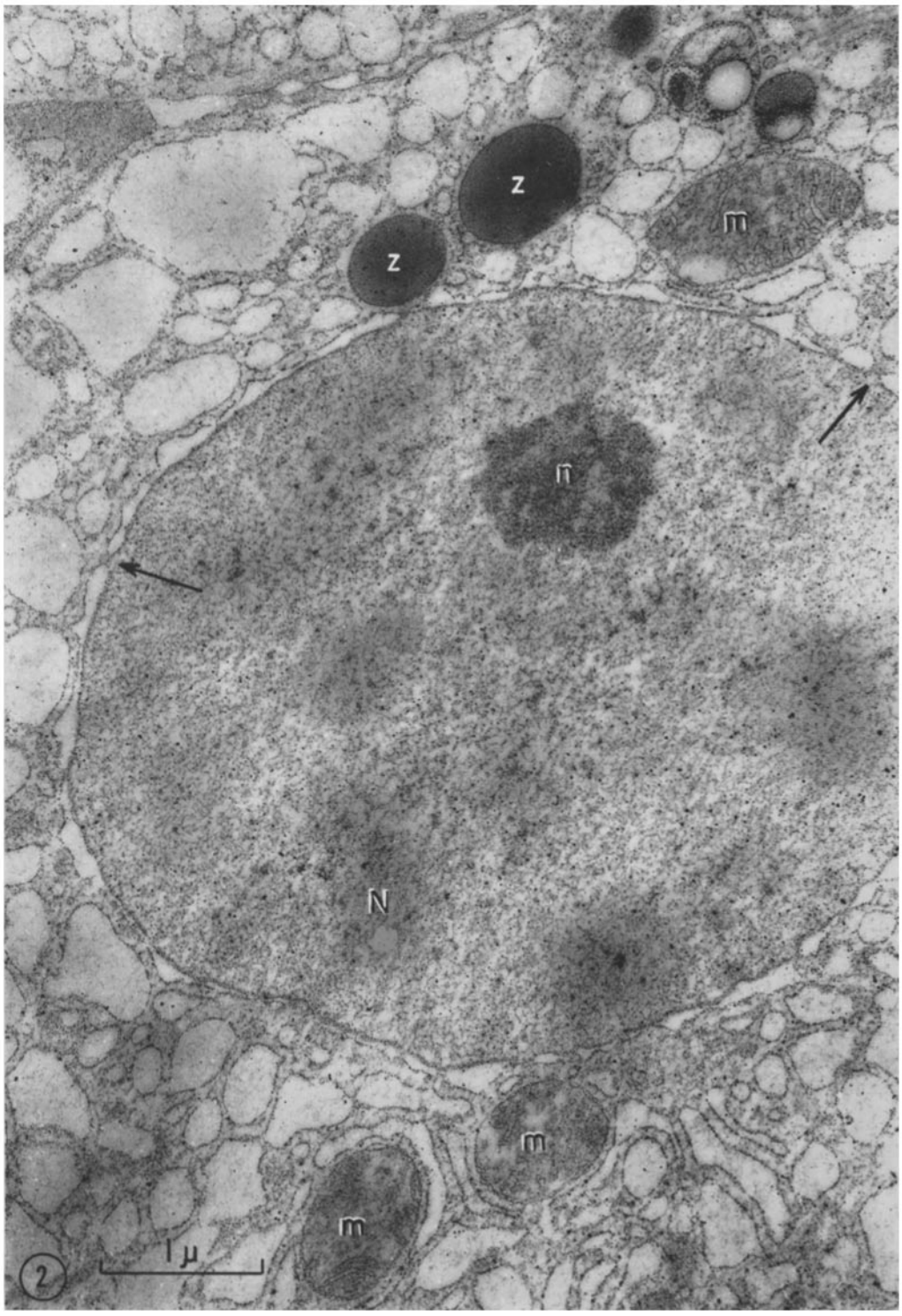


FIG. 1

by means of a platinum wire loop. In order to avoid adhesion of the sections to the walls of the Petri dish and their subsequent loss during the change of reagents, they were placed within a hole cut in the center of a thin disk of plastic foil (mylar) which was kept floating on the water. A notch at the border of the disk permitted the introduction of the tip of a pipette for the change of reagents (Fig. 1A). After aspirating the water, the Petri dish was filled with an ammoniacal silver solution and placed in an oven at 50°C. for 20 to 30 minutes. The solution was prepared by mixing 0.25 per cent silver nitrate, 10 ml., methenanime 0.30 mg.; 5 per cent borax, 8 ml.; the volume being finally brought up to 30 ml. with distilled water. After impregnation, the ammoniacal silver solution was allowed to cool completely, and then removed by aspiration and replaced by several changes of distilled water for washing. Treatment with

FIGURE 2

Acinar cell of human pancreas after 25 minutes staining (at 50°C.) in ammoniacal silver solution. Silver granules (100 A size) are linearly arranged on the ergastoplasmic and mitochondrial (*m*) membranes. The membranes bordering the zymogen granules (*Z*) are made particularly evident by the silver impregnation. A few silver granules are deposited in the mitochondrial matrix and in the zymogen granules; in these latter the silver particles tend to form small conglomerates. The arrows indicate the pores in the nuclear envelope. *N:* nucleus. *n:* nucleolus. × 24,000.


z
z
m
n
N
m
m
1 μ
2

a 0.5 per cent thiosulfate solution for about 1 minute, followed by a last washing in distilled water, completed the procedure.

At this point a formvar-coated copper grid was allowed to fall upon each ribbon section, in such a way that the formvar film would come into contact with the sections (Fig. 1B). Each copper grid (with its respective sections) was then picked up with a platinum wire loop (Fig. 1C) and placed by turning the loop upside down (Fig. 1D) upon the end of a small cylinder made up of filter paper, and held upright in a plastic rack. The filter paper cylinders had about the same diameter as the wire loop, and were made by rolling filter paper tightly and securing the roll with scotch tape.

Some sections were treated with a 1 per cent periodic acid solution for 15 to 20 minutes prior to silver impregnation which, in some cases, was extended up to 1 hour.

Other sections were treated with ammoniacal silver solution after oxidation (carried out for 15 to 60 minutes at room temperature) with a solution containing 15 per cent hydrogen peroxide and 0.6 N HCl, followed by treatment with a dilute solution of oxalic acid and washing in distilled water.

RESULTS

1. *Silver Impregnation of Sections neither Treated with Xylol nor Previously Oxidized with Periodic Acid*

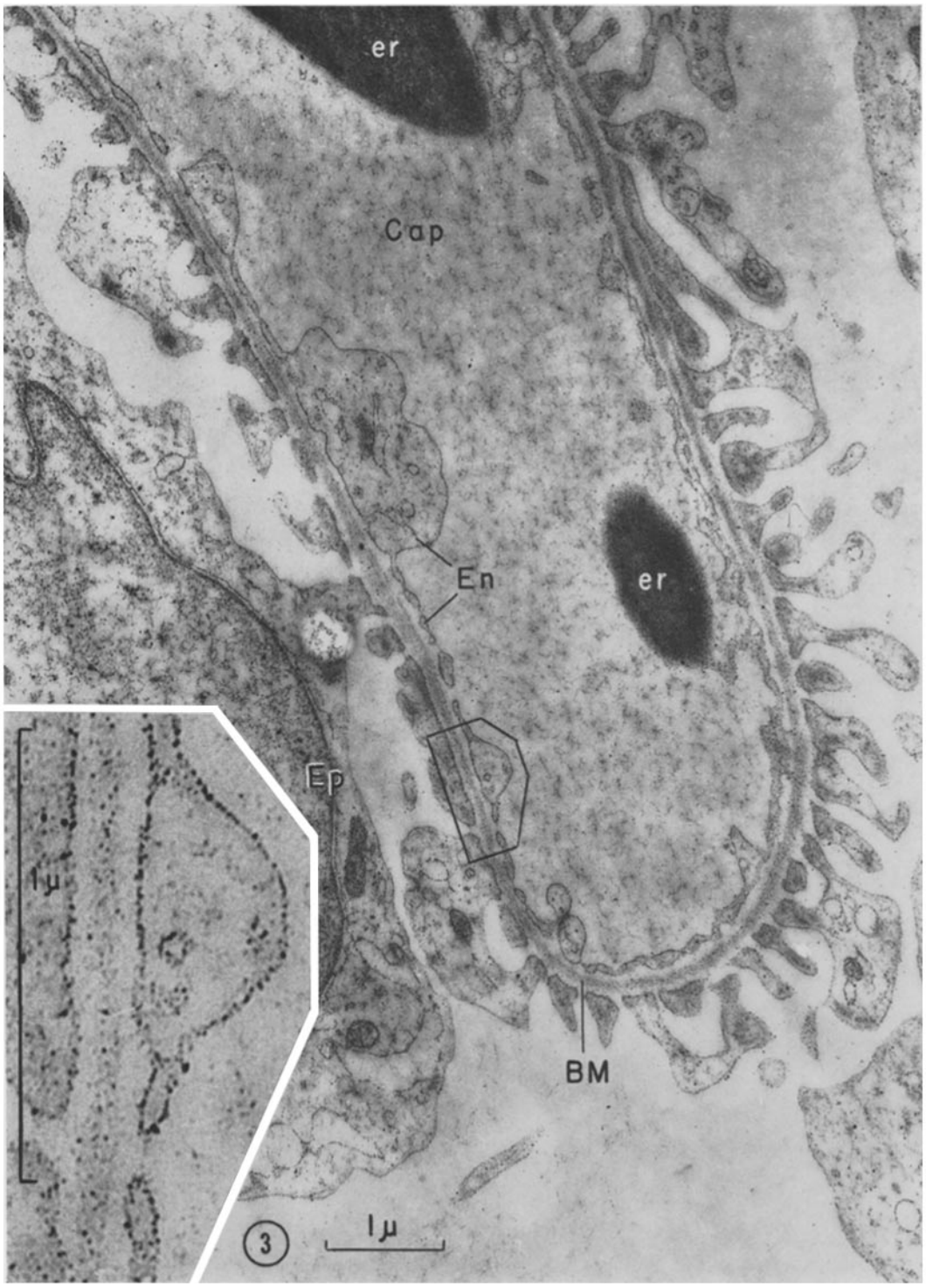
The most remarkable result of this technique is an enhanced contrast of all the principal tissue structures which is obtained without appreciable distortion. As a consequence, the technique can be used to facilitate electron microscopic observations at low magnifications. The silver granules have an average diameter of 100 A, and appear deposited along the cut edge of all membranous cytoplasmic structures, including ergastoplasmic membranes, as well as mitochondrial cristae (Figs. 2 to 7). A few silver granules are also found deposited within the cytoplasmic matrix, where—in spite of their higher density—they are not easily distinguishable from Palade's granules. In certain regions of the cytoplasm, which normally exhibit a high density, *e.g.* the extremity of the foot processes of the epithelial cells of the renal glomerulus, a particularly conspicuous deposit of silver granules is found (Fig. 3); however, the random distribution of the silver granules in these cases does not suggest the existence of an organized substrate. A similar situation is encountered in the nucleus and nucleolus, although sometimes the silver granules tend to form a more or less distinct network within the nucleus (Fig. 2). The two perinuclear membranes are also particularly evident after silver impregnation (Figs. 2 and 3). In exocrine pancreatic cells, the continuity of the two membranes is clearly seen at the level of the pores (Fig. 2). Continuity between the nucleoplasm and the cytoplasmic matrix surrounding the ergastoplasmic sacs seems to be established across these pores which have an average diameter of 500 A.

Fig. 3 demonstrates that silver is also deposited upon the basement membranes of the renal glomerular capillaries. The distribution of the granules within this basement membrane is more or less uniform and their concentration is directly proportional to the duration of the treatment with the ammoniacal silver solution and to the temperature of the latter. Red blood cells (Fig. 3) and elastic fibers display a high argentaffinity,

FIGURE 3

Glomerular capillary of rat kidney after 20 minutes staining (at 55°C.) in ammoniacal silver solution. Silver impregnation increases the contrast of cytomembranes of epithelial (*Ep*) and endothelial (*En*) cells. Silver deposition is heaviest in the foot process of epithelial cells. The distribution of the silver granules is almost homogeneous in the basement membrane (*BM*) of capillary loops. Silver deposition upon erythrocytes (*er*) and precipitated plasma content of the capillary lumen is particularly pronounced. × 18,000.

Lower left. An enlargement of the area enclosed in the rectangle to show granular silver deposits on the cytoplasmic membrane of endothelial cell and basement membrane. × 72,000.


er
Cap
En
er
Ep
1μ
BM
3
1μ

but the distribution of the silver granules therein is apparently random.

In collagen fibers, the silver granules are arranged in transverse rows each containing about ten granules, the distance between each granular stripe being about 600 A, a fact which evidences the characteristic periodic structures of these fibers.

2. *Silver Impregnation of Sections Treated with Xylol but not Previously Oxidized with Periodic Acid*

The results are very similar to those above described, the only difference being a decreased deposit of silver upon cytomembranes (Fig. 10) and in red blood cells.

3. *Silver Impregnation of Sections Previously Oxidized with Periodic Acid*

After oxidation of the sectioned tissue with periodic acid, there is not appreciable silver deposition upon protoplasmatic structures (Fig. 8), but the behavior of basement membranes, collagen, and elastic fibers is not greatly affected. Only the amount of deposited silver appears to be slightly reduced in basement membranes and collagen fibers (Fig. 9). When treatment with silver ammoniacal solution is sufficiently prolonged, the deposition of silver upon and within metaplasmic structures is considerably increased (Figs. 11 to 13), whereas protoplasmic structures do not appear more heavily impregnated.

4. *Silver Impregnation of Sections After Bleaching with Hydrogen Peroxide*

After the oxidation of the sections with hydrogen peroxide, silver can no longer impregnate protoplasmic components. The behavior of metaplasmic structures is highly variable, and depends on the duration of the treatment with hydrogen peroxide.

DISCUSSION

The demonstration that it is possible to impregnate with silver thin sections, without removing the embedding methacrylate, or resorting to special embedding methods that give sections permeable to water (17), is the salient point of the present work. The methacrylate in no way conditions the mode of silver deposition, since experimental evidence proves that the silver particles are deposited in the same fashion when impregnation is carried out before embedding (10, 11, 18). There remains to be proved whether the impregnation obtained in sections is limited to a surface reaction or results from the actual penetration of silver particles within the section. Should the second hypothesis be true, then one might consider that the probable displacement of the silver particles, which occurs during the sublimation of the methacrylate under the electron beam, causes the distortion of the structures with which the silver particles are associated.

The comparative analysis of the results obtained by impregnating under the various experimental conditions mentioned, suggests the following conclusions:

1. The cytomembranes display a high degree of primary argentaffinity when tissues are fixed with OsO_4.

2. When OsO_4-fixed tissues are treated with xylol, the argentaffinity of the cytomembranes is selectively reduced.

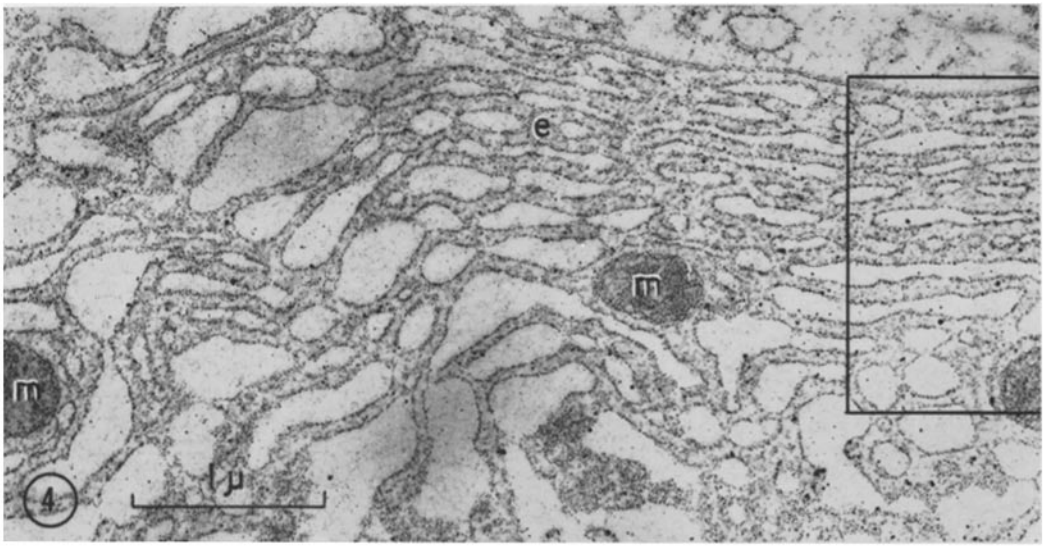
3. Bleaching of the sections with hydrogen peroxide removes the argentaffinity of all tissue components.

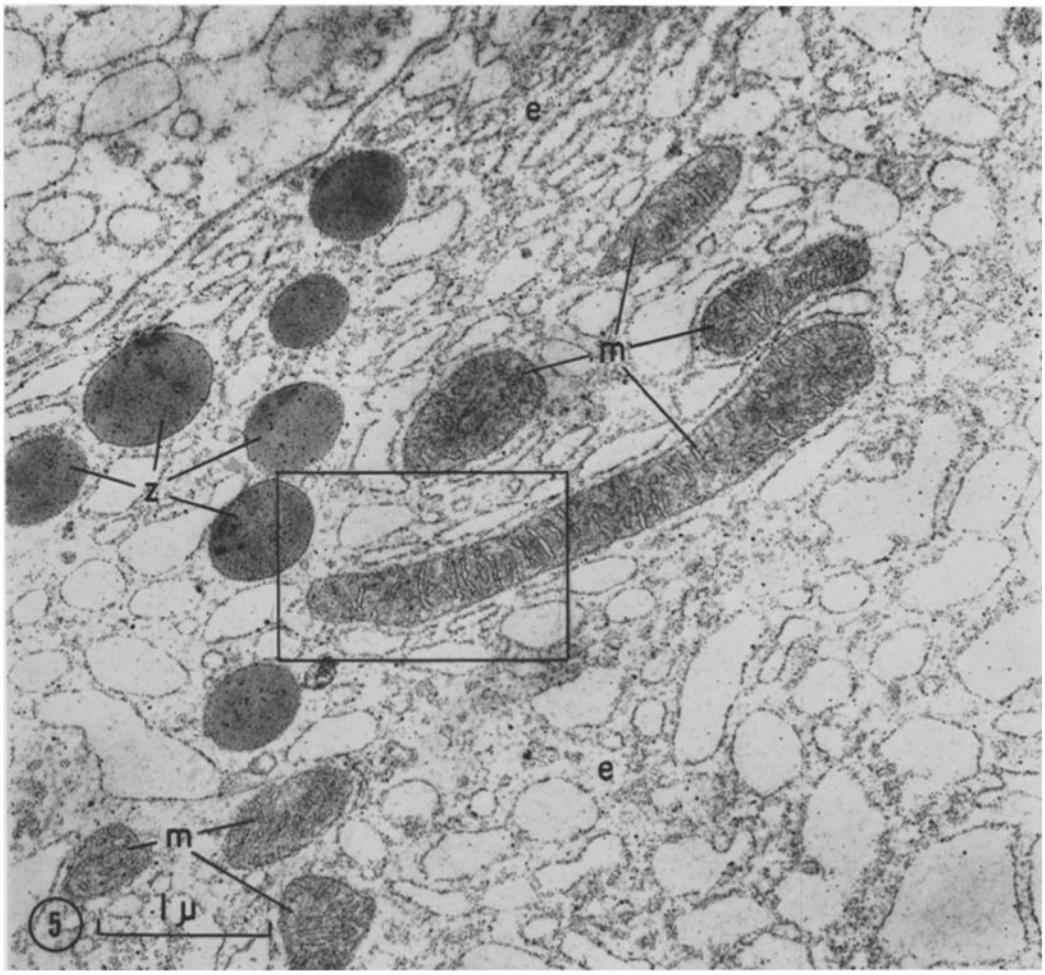
FIGURES 4 and 5

Acinar cell of human pancreas after 25 minutes staining (at 50°C.) in ammoniacal silver solution. In spite of the granularity of the silver deposits, the cristae of the mitochondria and the two membranes enveloping them are clearly evident. A few silver granules are located between the ergastoplasmic cisternae (*e*); however, they are scarcer than the RNP particles. *m*: mitochondria. *z*: zymogen.

Fig. 4, × 25,000.

Fig. 5, × 23,000.


e
m
m
4
1μ


e
m
z
e
m
5
1μ

4. Oxidation of the sections with periodic acid destroys the argentaffinity of protoplasmic structures, but does not affect that of metaplasmic structures.

As regards the chemical nature of the impregnated structures, it is generally assumed, on the one hand, that most of the Os contained in OsO_4-fixed tissues is bound to unsaturated lipids as lower Os oxides; on the other hand, it is currently believed that a major fraction of the unsaturated lipids is contained in cytomembranes (19).

It is also known that when sections of OsO_4-fixed tissue are treated with oxidizing agents, such as hydrogen peroxide, Os can be removed by making it water soluble in the tetroxide form (20). One may justly presume that analogous effects might be produced by oxidation with periodic acid. The lack of silver impregnation of protoplasmic structures after periodic acid or hydrogen peroxide treatment would seem to demonstrate (by a similar reasoning) that their argentaffinity is bound to the presence of reduced osmium.

The argentaffinity of basement membranes and collagen fibers after periodic acid treatment may be explained, according to the interpretation generally given to Gomori's method, as resulting from the reduction of the ammoniacal silver solution by free aldehyde groups derived from mucopolysaccharides (21). The same explanation does not apply, however, to the successful silver impregnation of various structures in OsO_4-fixed tissues not subsequently oxidized with periodic acid. In this case it is not improbable that silver reduction is brought about by the reduced osmium present in metaplasmic structures.

The argentaffinity of the elastic tissue, which is not substantially modified by periodic acid treatment, may be reasonably explained as resulting from silver reduction by some reducing agents produced by OsO_4 reaction with cementing substances. Further studies, however, are required to elucidate this point.

According to what has been said above, one may explain the decreased cytomembrane argentaffinity after treatment with xylol as the result of lipid extraction brought about by the solvent action of xylol, and followed by subsequent release of bound osmium. However, this hypothesis must be supported by more extensive research, since it is in opposition with the results of other workers (6, 19) according to whom the lipids are no longer soluble in ordinary organic solvents after reaction with OsO_4.

I wish to express my gratitude to Mr. Tomassini, technician of Istituto Superiore di Sanità, for the valuable collaboration given to me during this research.

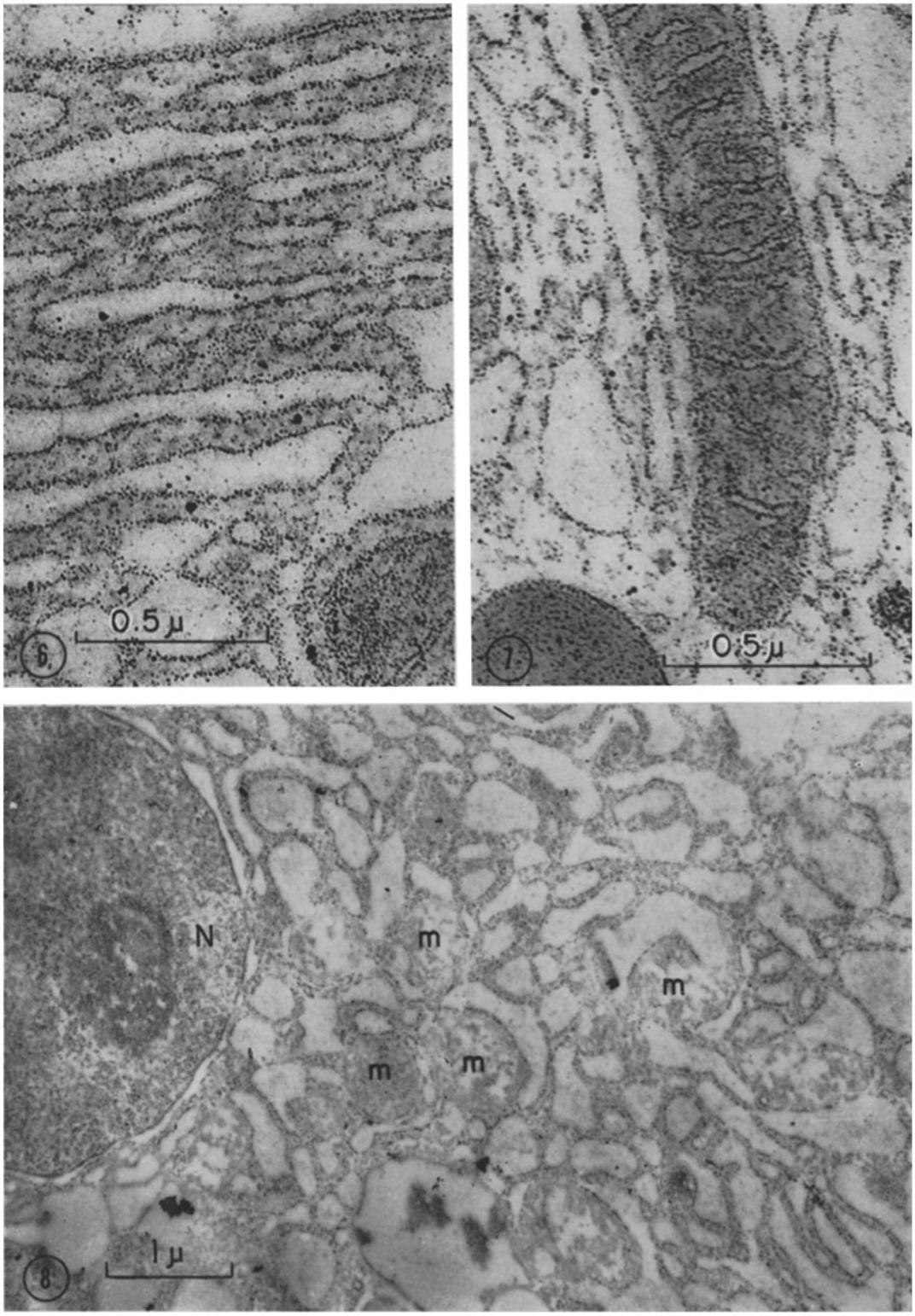
FIGURES 6 and 7

Enlargements of the areas enclosed by rectangles in Figs. 4 and 5 to demonstrate details of the ergastoplasmic system of the mitochondrial membranes and of zymogen granules.

Fig. 6, × 60,000.
Fig. 7, × 64,000.

FIGURE 8

Acinar cell of human pancreas after oxidation with 1 per cent periodic acid followed by silver impregnation (20 minutes at 55°C.). There is no silver deposition on the protoplasmic structures. The contrast of the mitochondrial membranes is greatly reduced. *n:* nucleus. × 19,000.


0.5 μ
6
0.5 μ
7
N
m
m
m
m
1 μ
8

BIBLIOGRAPHY

1. HUXLEY, H. E., Electron microscope studies of the organisation of the filaments in striated muscle, *Biochim. et Biophysica Acta*, 1953, **12**, 387.
2. JAKUS, M. A., Studies on the cornea. II. The fine structure of Descemet's membrane, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 243.
3. HUXLEY, H. E., The double array of filaments in cross-striated muscle, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 631.
4. HODGE, A. J., Studies on the structure of muscle. III. Phase contrast and electron microscopy of Dipteran flight muscle, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 361.
5. ROBERTSON, J. D., The ultrastructure of adult vertebrate peripheral myelinated nerve fibers in relation to myelinogenesis, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 271.
6. FERNÁNDEZ-MORÁN, H., and FINEAN, J. B., Electron microscope and low-angle x-ray diffraction studies of the nerve myelin sheath, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 725.
7. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.
8. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 727.
9. LACY, D., and CHALLICE, C. E., Mitochondria and other cytoplasmic inclusions, *Symp. Soc. Exp. Biol.*, No. 10, Cambridge University Press, 1957, 62.
10. WASSERMANN, F., and KUBOTA, L., Observations on fibrillogenesis in the connective tissue of

FIGURE 9

A portion of a glomerular capillary loop of the rat kidney after oxidation with 1 per cent periodic acid followed by silver impregnation (20 minutes at 55°C.). Silver deposition is not appreciable in either cytoplasmic structures or basement membrane (*BM*). (Compare to Figs. 12 and 13). × 16,500.

FIGURE 10

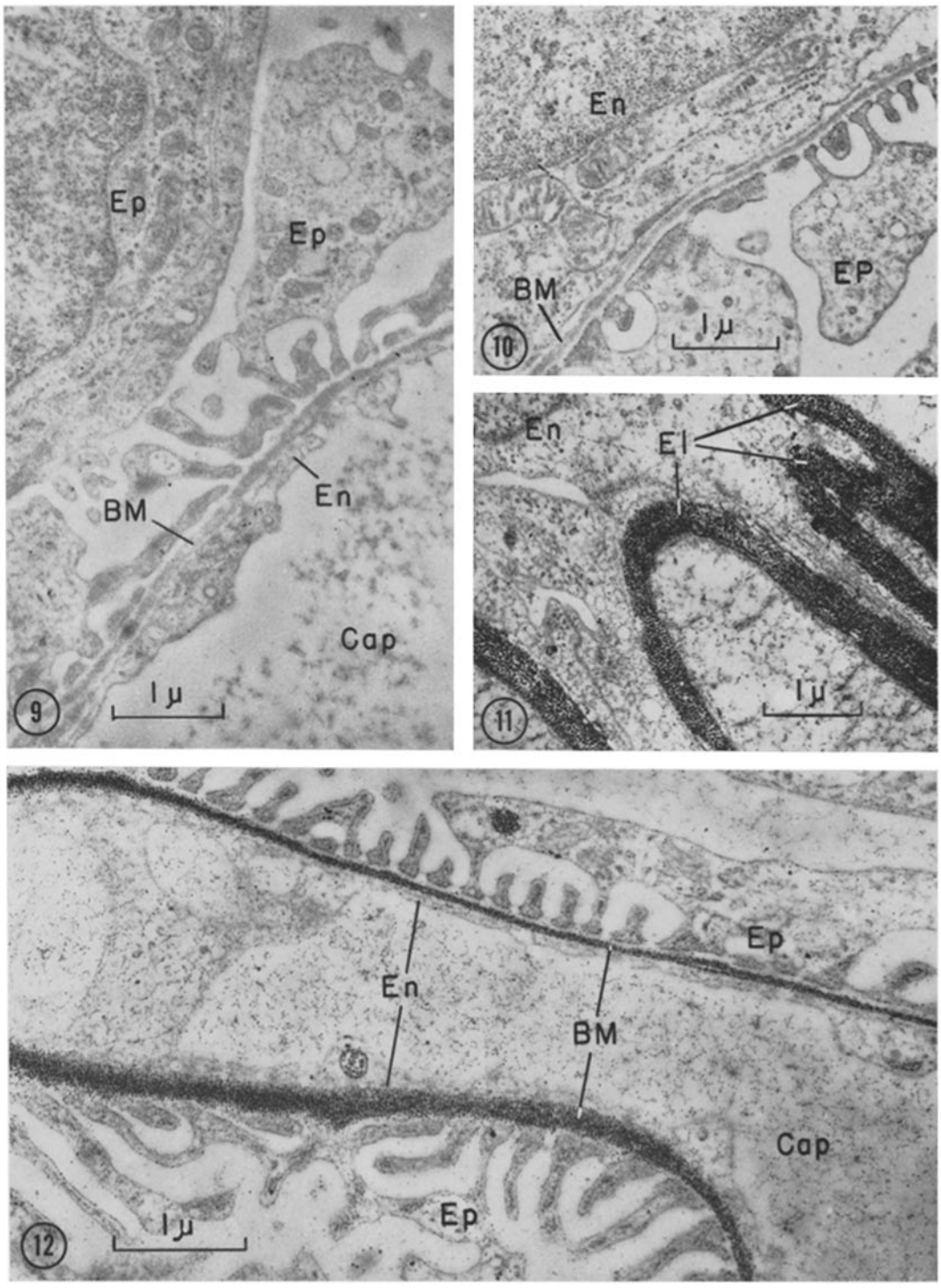
A portion of a glomerular capillary loop of the rat kidney. The tissue block was treated with xylol before embedding in methacrylate and the sections were stained in ammoniacal silver solution for 20 minutes at 55°C. Silver impregnation of the cytomembranes of epithelial (*Ep*) and endothelial (*En*) cells is greatly reduced relative to that in sections not previously treated with xylol (see Fig. 2). × 16,000.

FIGURE 11

Afferent arteriole of rat kidney after oxidation with 1 per cent periodic acid followed by silver impregnation (1 hour at 55°C.). Silver deposits are particularly conspicuous on the internal elastic membrane (*EI*). Here silver particles tend to conglomerate in larger granules with almost even distribution throughout the elastica. Silver particles scattered in the cytoplasm of the endothelial cell (*En*) are not selectively associated with membranous structures. × 14,500.

FIGURE 12

A peripheral portion of rat kidney glomerulus stained with ammoniacal silver solution for 1 hour at 55°C. after oxidation with 1 per cent periodic acid. Granular appearance of the silver deposits upon the basement membrane. Silver impregnation of the cytomembranes of epithelial (*Ep*) and endothelial (*En*) cells is negligible. The foot processes of epithelial cells contain a few linearly arranged silver granules along their axis. × 20,000.


Ep
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10
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El
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11
Ep
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Cap
Ep
1 μ
12

the chick embryo with the aid of silver impregnation, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 67.

11. CHURG, J., MAUTNER, W., and GRISHMAN, E., Silver impregnation for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 841.
12. MARINOZZI, V., La struttura dell'alveolo polmonare indagata con una particolare tecnica che permette l'utilizzazione delle sezioni ultrasottili anche per l'osservazione al microscopio ottico, *Atti Accad. Lincei*, 1958, **24**, 600.
13. MARINOZZI, V., Nuovi rilievi sulla fine struttura degli alveoli polmonari desunti dall'osservazione al microscopio elettronico di sezioni ultrasottili sottoposte ad impregnazione argentica, *Atti Accad. Lincei*, 1958, **24**, 754.
14. MARINOZZI, V., Colorazione delle sezioni ultrasottili per il microscopio elettronico per mezzo dell'impregnazione argentica, *Atti Accad. Lincei*, 1958, **25**, 1.
15. MARINOZZI, V., Coloration de coupes ultra-fines pour la microscopie électronique au moyen de l'imprégnation a l'argent, *4th Internat. Kong. Elektronenmikr.*, Berlin, Springer-Verlag, 1958.
16. JONES, B. D., Nephrotic glomerulonephritis, *Am. J. Path.*, 1957, **33**, 313.
17. GRIGG, W., and HOFFMAN, H., A double imbedding method giving water-permeable ultrathin sections for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 331.
18. DETTMER, N., NECKEL, I., and RUSKA, H., Elektronenmikroskopische Befunde an versilberten Kollagenen Fibrillen, *Z. wissensch. Mikr.*, 1961, **60**, 290.
19. BAHR, G. F., and ZEITLER, E., Image interpretation and contrast in thin sections, *Scient. Instruments*, 1959, **4**, 1.
20. MERRIAM, R. W., The contribution of lower oxides of osmium to the density of biological specimen in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 579.
21. LILLIE, R. D., Argentaffin and Schiff reaction after periodic acid oxidation and aldehyde blocking reactions, *J. Histochem. and Cytochem.*, 1954, **2**, 127.

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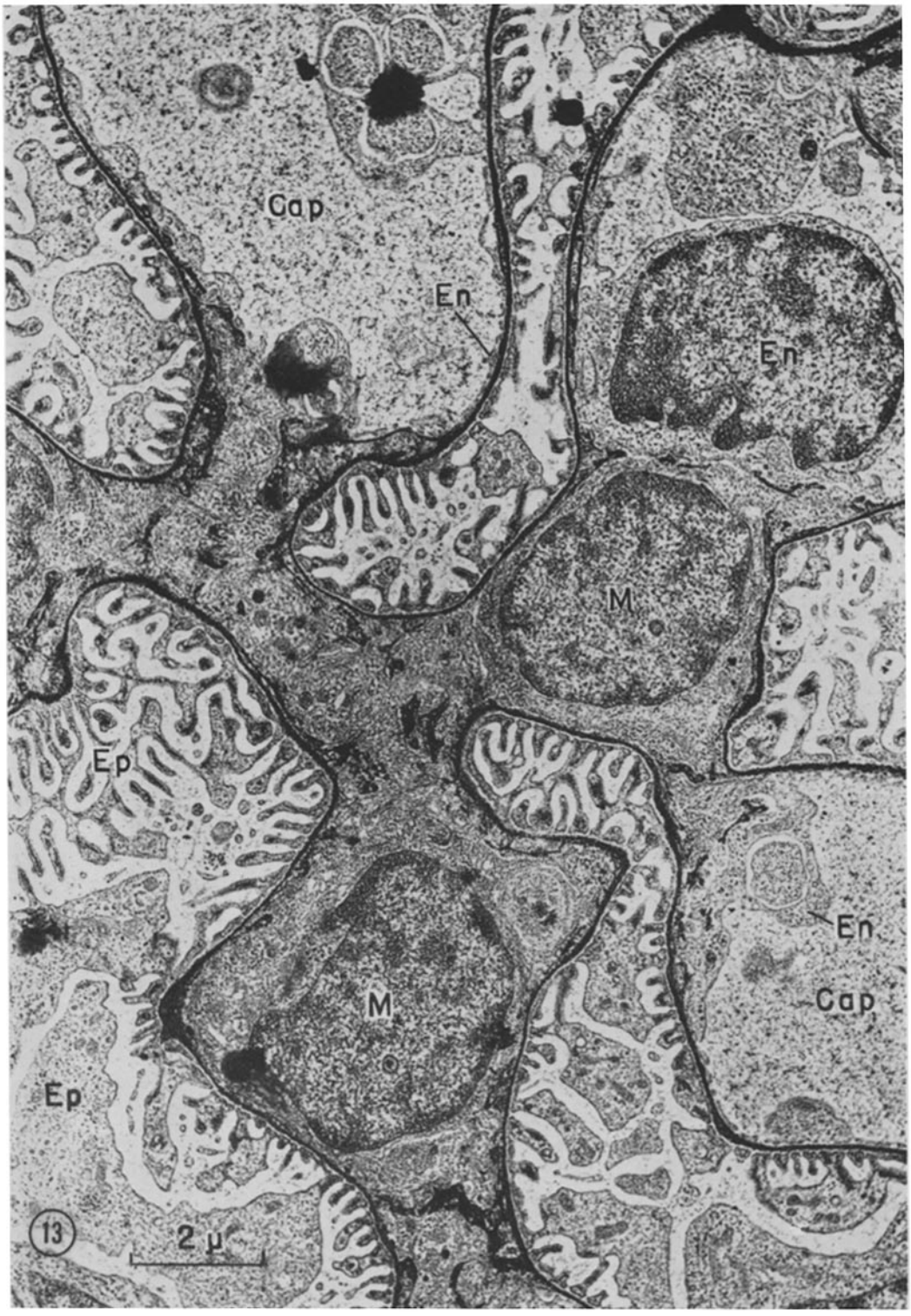
FIGURE 13

A central portion of the same glomerulus. The basement membrane exhibits the highest contrast. Mesangium cells (*M*) are enmeshed in a sponge-like framework formed by basement membrane sheets of varying thickness. A perforated laminar expansion of basement membrane is interposed between endothelial (*En*) and intercapillary cells (*M*). × 10,000.


Cap
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