THE USE OF SILVER NITRATE AS A VITAL STAIN, AND ITS DISTRIBUTION IN SEVERAL MAMMALIAN TISSUES AS STUDIED WITH THE ELECTRON MICROSCOPE*

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INTRODUCTION

Dyestuffs have been administered to living animals for many reasons. Ehrlich (1885), using dyes which change color upon oxidation or reduction, attempted to determine oxidative potentials in animal tissues. Basic dyes are, in general, quite toxic for reasons not clearly understood. A group of acid, colloidal dyes, such as pyrrol blue, trypan blue, and carmine, were shown by Goldmann (1909), Kiyono (1914), and Evans and Scott (1921), to be segregated by phagocytosis into a class of scavenger cells, the macrophages. These and related dyes, as well as numerous opaque or colored particulate materials (India ink, thorotrast, manganese dioxide, colloidal gold, etc.), have been used in many studies designed to investigate the phagocytic activity of cells and the passage of fluid across cellular membranes, and to mark cells in order to follow their subsequent migration by the colored inclusions.

The introduction of silver salts into animals causes a deposition of metallic silver in many tissues and organs. Clinically, this phenomenon is called argyria, which often follows accidental ingestion of silver from solutions such as argyrol, or the absorption of silver as an industrial hazard. In argyria, the skin, mucous membranes, and many organs become darkened and discolored because of their content of finely dispersed granules of silver metal. The location of the silver has been studied carefully by microscopical methods, so that quite complete accounts of the histopathological lesions in argyria are available (Hill and Pillsbury, 1939). More recently, Gatz (1949) has shown that the administration of very dilute solutions of silver nitrate in drinking water is tolerated with no visible difficulty by rats, and that after many months of such treatment the tissues become heavily laden with silver deposits. This procedure, which permits segregation of the silver without its

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reaching toxic concentrations, has been exploited by Wislocki and Leduc (1952), who found that observation of brain sections with both daylight and darkfield microscopes permitted a quite precise delimitation of the blood-brain barrier at the margins of those areas of the brain in which vital staining occurs. Their success with darkfield illumination, which reveals silver particles too small for visualization by ordinary microscopy, prompted us to study the distribution of silver in tissues examined with the electron microscope. We hoped the greater resolution afforded by this instrument would permit more precise localization of the silver deposits. The following passages will illustrate that this hope was well founded, and will describe the ultramicroscopic distribution of silver in the cells and their associated membranes in a number of tissues.

Materials and Methods

Two Swiss albino mice, four albino rats, and two guinea pigs were used. The mice and rats received 1 gm. of $AgNO_3$ per liter in their drinking water for 6 to 12 months. The guinea pigs were given 0.01 per cent $AgNO_3$ for 1 and 2 months, respectively. At the time of autopsy all animals appeared to be healthy. The skin and lips were discolored, and the internal organs were variably darkened by their silver deposits.

The animals were killed by decapitation, the tissues were removed rapidly and small pieces were immersed in Palade's (1952 *a*) buffered osmic acid mixture. After fixation at room temperature for 4 hours the tissues were dehydrated, infiltrated with a 3:1 mixture of butyl and methyl methacrylates, and finally embedded in this mixture of plastics by polymerization catalyzed by benzoyl peroxide. Sections were prepared as described by Dempsey and Lansing (1953) and examined by an RCA electron microscope model EMU at initial magnifications of one to six thousand diameters. The negatives obtained were enlarged photographically as desired. The tissues employed in this study were the kidney, pancreas, liver, and thyroid gland.

RESULTS

Kidney.—The deposits of silver, as visualized in electron micrographs, appeared as dense, granular particles of irregular shapes and sizes. In places, where heavy deposition had occurred, the aggregated granules had diameters up to a few microns; in others, where slight amounts of silver were present, the granules were smaller. Indeed, in our best micrographs, the smallest discernible particles were as small as the limits of resolution of the microscope (in the order of 20 to 30 A). The particles, of somewhat larger size, were easily identifiable because of their extreme density and their sharp angular outlines. No structures even remotely resembling them have ever been seen in several thousand micrographs from various mammalian tissues prepared identically except that silver had not been administered.

The heaviest deposits of silver were located in the basement membranes of the kidney. The glomerular basement membranes were most heavily infiltrated with silver; next in order were the membranes surrounding the

proximal convoluted tubules. The basement membranes of the distal convoluted tubules contained only occasional granules (Figs. 1 and 2).

The localization of the granules in the basement membranes was quite precise. In the glomerulus, silver was not detectable in the endothelial cells lining the capillaries and only rarely in the epithelium forming the visceral layer of Bowman's capsule. The latter observation is interesting, because the epithelial pericytes exhibited curious foot-like processes directed toward the glomerular basement membrane upon which they rested. Between these extensions minute spaces occurred, which communicated with the lumen of the glomerular cavity. These foot-like cytoplasmic extensions have been recognized only in electron micrographs, and in the earlier pictures made from thick sections with poor resolution there was some doubt concerning their exact relationships to the pericytes and basement membranes (Pease and Baker, 1950). Others (*cf.* Rinehart *et al.*, 1953) have traced these processes to the pericytes. In our current pictures (Fig. 3), where a sharp localization of silver is visible in the glomerular basement membrane but not in the end-feet, the conclusion that the end-feet are processes of the pericytes is inescapable.

The cells of the parietal layer of Bowman's capsule were devoid of silver, but the basement membranes associated with them contained moderate numbers of small granules (Fig. 1).

In the proximal convoluted tubules, identifiable by their characteristic brush border at the luminal surface, a moderate amount of silver was deposited in the basement membrane. Figs. 2 and 4 illustrate this arrangement, showing the interesting relationship between the basement membrane and the cellular plasma membrane. This basement membrane apparently forms an essentially smooth, flat sheet enclosing the renal tubule (Fig. 4). The bases of the epithelial cells are composed of a series of complex cytoplasmic evaginations or invaginations, the contours of which are visualized by the denser shadows of the cellular plasma membrane (Figs. 2 and 4). Thus, between the folds of the plasma membrane, there are extracellular pockets into which the basement membrane does not appear to extend. The granules of silver are located in the basement membrane and not in the numerous pockets between the folds of the cell membranes (Fig. 4).

The mitochondria of the cells comprising the proximal convoluted tubules were relatively large cylindrical, or filamentous structures. In high resolution micrographs they were seen to be bounded by an outer membrane, closely apposed to an inner membrane which was often reflected into folds projecting into the interior of the mitochondrion. A rather dense, homogeneous material filled the central cavity between the folds. This appearance was entirely similar to that described by Palade (1952 *b*, 1953), Sjöstrand and Rhodin (1953), and Rhodin (1954). However, in our animals which

stored silver nitrate, many (approximately one-fourth) of the mitochondria exhibited one or more dense, spherical granules embedded in the central matrix. These granules were ordinarily solitary and had a diameter of about 0.1 μ (Figs. 2 and 4). Since the ratio of diameters of granules and mitochondria is about 1:4, it would seem likely that each mitochondrion contains at least one such granule; those not exhibiting a granule in our pictures would be explained by the plane of section missing the granule. Mitochondrial granules have been reported by Palade (1952 *b*) and Rhodin (1954) but the frequency and the density of those encountered here appear to us to be greater than are encountered in the tissues from untreated animals.

In the distal convoluted tubules, silver was encountered only in small amounts in the basement membranes (Fig. 1), and no mitochondrial granules were observed.

Silver was encountered in occasional macrophages in the peritubular stroma, in the form of silver granules of varying size and irregular shape within cytoplasmic vacuoles. It also occurred in the basement membranes of the intertubular capillaries, but none was visible in the endothelium.

Pancreas.—Figs. 5, 6, 7, and 10 illustrate the localization of silver in the pancreatic acinar cells, basement membranes, blood vessels, and intralobular ducts. We have not encountered islands of Langerhans nor the larger ducts and so have no observations to offer concerning these structures.

The heaviest concentrations of silver granules were found in the basement membranes underlying the endothelium of small arterioles. The basement membranes of capillaries (Fig. 6) and small intralobular ducts (Fig. 5) also exhibited numerous silver granules. Occasional macrophages were encountered in the interlobular connective tissue; these contained large segregation vacuoles filled with granular deposits of silver (Fig. 11).

Perhaps the most interesting locus in which silver was found in the pancreas consisted of occasional deposits within the acinar cells. These deposits appeared as rather sizable aggregates of fine granules, the largest groups of which measured 2 to 3 μ in diameter. These were encountered in all parts of the cell, but occurred most frequently in the zone just apical to the nucleus (Fig. 7). In some of these aggregates, the silver was packed so tightly that no other structure could be seen. In others, however, the silver granules were located inside a spheroidal structure bounded by a dark membrane and containing, besides the granules, a homogeneous substance the density of which was comparable to that of mitochondria. In still others, in which only small silver granules occurred sparsely, the spheroidal structures exhibited the internal folds characteristic of mitochondria (Figs. 9 and 10).

Liver.—Silver deposits were encountered in the parenchymatous cells of the liver and in the lining cells of the hepatic sinusoids (Kupffer cells). We

have not investigated the vessels, ducts, and connective tissues located in the portal canals.

The Kupffer cells exhibited vacuoles containing granular aggregates similar to those described in preceding sections for macrophages (Figs. 8 and 11). Our specimens illustrate, incidentally, that the hepatic sinusoids are incompletely lined by the Kupffer cells, a phenomenon previously mentioned by Fawcett (1953). Here and there are locations in which the hepatic cells seem to be bathed directly by the blood plasma within the sinusoid. This disposition would seem to be a true one and not the result of a mechanical artefact, since the Kupffer cells show no evidence indicative of shrinkage and small irregular surface projections of the hepatic cells dip into the lumen of the sinusoid. These appearances suggest that an incomplete layer of lining cells is interposed between the blood stream and the hepatic cells.

The hepatic cells exhibited occasional aggregates of granules contained within vacuolated structures (Fig. 9). These intracellular aggregates occurred more frequently than did the similar ones described for the pancreas. Like those in the pancreas, those with small amounts of silver often showed internal folds, the double membranes of which had dimensions equal to those of mitochondria. In favorable places, the limiting outer membrane could also be resolved into a double layer similar to that of mitochondria. In bodies with slight deposits, the silver was located on the internal membranes, whereas in ones with heavier deposits there were vesicles filled with granules of varying sizes.

Thyroid Gland.—Silver deposits occurred in the basement membranes of the thyroid follicules, and in the similar membrane supporting the endothelial cells of the capillaries. The depiction of the follicular basement membrane is interesting, because the existence of such a membrane has been denied by many histologists. It appears, however, that the thyroid cells rest upon a structure identical in appearance and ability to attract silver granules with that forming the basement membrane of other epithelia. Occasional macrophages, with their segregation vacuoles, were encountered in the interfollicular connective tissue. Silver was not encountered elsewhere in the thyroid gland.

DISCUSSION

The discovery that certain acid, colloidal dyes could be administered to animals with minimal toxicity led to the investigation of the phagocytic activity of cells of the body, to the recognition of the free and fixed macrophages, and to some understanding of the mechanisms whereby cells segregate or otherwise handle unassimilable colloidal materials (Goldmann, 1909, 1912; Kiyono, 1914; Evans and Scott, 1921). The present study, dealing with the distribution of silver when used as a vital dye, introduces the electron micro-

scope as a tool for studying these and similar problems. Because of the greater resolving power of the electron microscope, more definite localization of the silver granules is possible than was the case with light microscopy. Thus, when formerly, with light microscopy, silver deposition was reported as occurring primarily in the glomerular endothelium, it is revealed with electron microscopy to be solely located in basement membrane (Gatz, 1949; Shaver and Mason, 1951). Furthermore, the distribution of silver in basement membranes and in the mitochondria of some cells can now be regarded as certain, when formerly one could say only that silver occurred within cells or apparently beneath their bases. Although the question was not thoroughly explored in the present investigation, it is evidently possible by means of this instrument to detect silver and other heavy metals in traces much beyond the resolution of the light microscope. The future promise of electron microscopy in these respects is great.

The visualization of silver after administering its salts also opens another field for investigation. Silver is a heavy metal, and, like other heavy metals is poisonous if toxic concentrations are reached. The density of the heavy metals renders them ideally suited for studies with the electron microscope. Detailed studies utilizing proper dosages, tissues, and heavy metal poisons should provide information as to the site at which these agents damage the cell. Our present findings, although exploratory only, are suggestive in that the mitochondria appear to be vulnerable to the heavy metal. The appearance of mitochondrial granules in the kidney and the deposit of silver upon the bounding and internal membranes of mitochondria from the kidney, pancreas, and liver suggest that these organelles are the targets of the heavy metal. Damage to mitochondria would provide a ready explanation for the toxicity of silver, since the mitochondria are now regarded as the sites of important oxidative activities.

The segregation of large amounts of silver in basement membranes in many locations, especially in pancreas, thyroid, salivary glands, Harderian glands, renal glomeruli, proximal convoluted tubules, urinary bladder, and chorioid plexuses (Hill and Pillsbury, 1939) suggests a possible function for these structures. Basement membranes, in general, are argyrophilic when sections of fixed tissues are exposed to solutions of silver salts. This argyrophilia, their positive reaction with the periodic acid–Schiff reagents, and the scanty information available from their analyses all point toward the presence of a polysaccharide in their composition. Since the silver administered in the present experiment was introduced in the ionic form and was reduced to metallic silver before its deposition, it is necessary to invoke some agent which has an adequate reducing activity. The carbohydrate moiety of basement membranes would appear to fulfill this requirement. Moreover, its location, being interposed between the blood stream and the parenchymatous

tissues, is so situated as to protect the tissues from exposure to toxic quantities of reducible substances, since these substances would be trapped and filtered out by the barrier of the membrane.

An alternative possibility is that the administered silver is quickly reduced in the gastrointestinal tract or in the blood stream, and that ultramicroscopic particles of colloidal silver are trapped in the basement membranes by a simple filtering action as fluids are transported across them. This possibility, although superficially simple, requires accessory explanations to account for the passage of particulate silver across the endothelial layer, for the aggregation of the small particles into larger and larger ones, and for the failure of silver to accumulate in extracellular spaces contiguous to the basement membrane such as the ones which we have noted in the renal proximal convoluted tubule. In any event, the observation that silver segregation occurs in sharply localized areas offers a challenge to discover the biophysical and biochemical mechanisms responsible, and provides a tool whereby basement membranes, mitochondria, and segregation vacuoles can be altered experimentally as a means of studying their biological significance.

SUMMARY

After chronic administration of a dilute solution of silver nitrate in drinking water to rats, mice, and guinea pigs, granular deposits of metallic silver were detected in electron micrographs of the kidney, liver, thyroid, and pancreas. The silver deposits were in the form of extremely dense, angular particles with sharp outlines. They varied from aggregates a few microns in diameter down to granules at the limit of resolution of the electron microscope.

The principal sites of deposition were (1) basement membranes, especially those of the renal glomeruli, proximal convoluted tubules, and various glands, and those associated with vascular endothelium, and (2) the cytoplasm of fixed and free macrophages. Both in Kupffer cells lining hepatic sinusoids and in the wandering macrophages of other tissues, the silver was segregated in discrete vacuoles. In addition, granular deposits were observed in occasional vesicular structures in the proximal convoluted tubules of the kidney, the hepatic cells, and the pancreatic acinar cell. These structures, in favorable preparations, contained an outer double layered membrane and internal folds similar to those of mitochondria, from which they appear to have been derived. The significance of these findings in heavy metal poisoning and in cellular physiology is briefly discussed.

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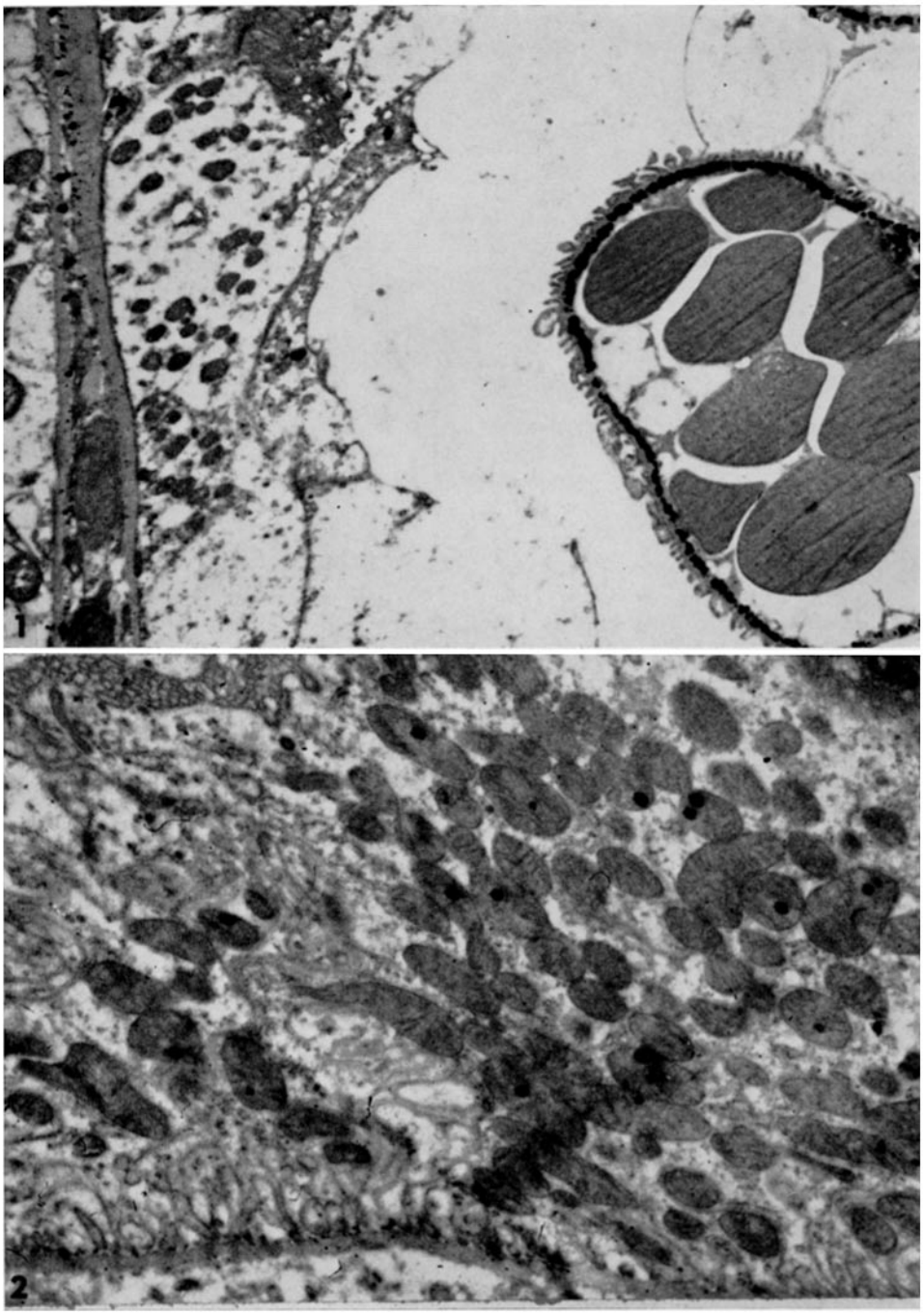
EXPLANATION OF PLATES

PLATE 31

FIG. 1. Section through the wall of Bowman's capsule and one loop of a capillary tuft from the renal glomerulus of a mouse which had received 0.0015 per cent $AgNO_3$ in its drinking water for 6 months. Heavy deposits of silver outline the basement membrane of the capillary on the right, and lesser deposits are seen in the basement membranes of Bowman's capsule and of a distal convoluted tubule on the left. Radiating into the glomerular lumen, some of the end-feet of the pericytes constituting the visceral layer of Bowman's capsule can be seen. × 5,000.

FIG. 2. Section through a proximal convoluted tubule from the kidney of a guinea pig which had received 0.01 per cent $AgNO_3$ in its drinking water for 6 weeks. The small amount of granular silver apparent in the basement membrane at the bottom of the figure is caused by the short time silver was administered. At the top left corner, a typical brush border can be seen. Dark granules occur in many of the mitochondria. At the base of the cell, many reflections of the plasma membrane upward into the cell's substance can be seen. The extracellular channels formed by these reflections do not exhibit silver deposits. × 15,000.

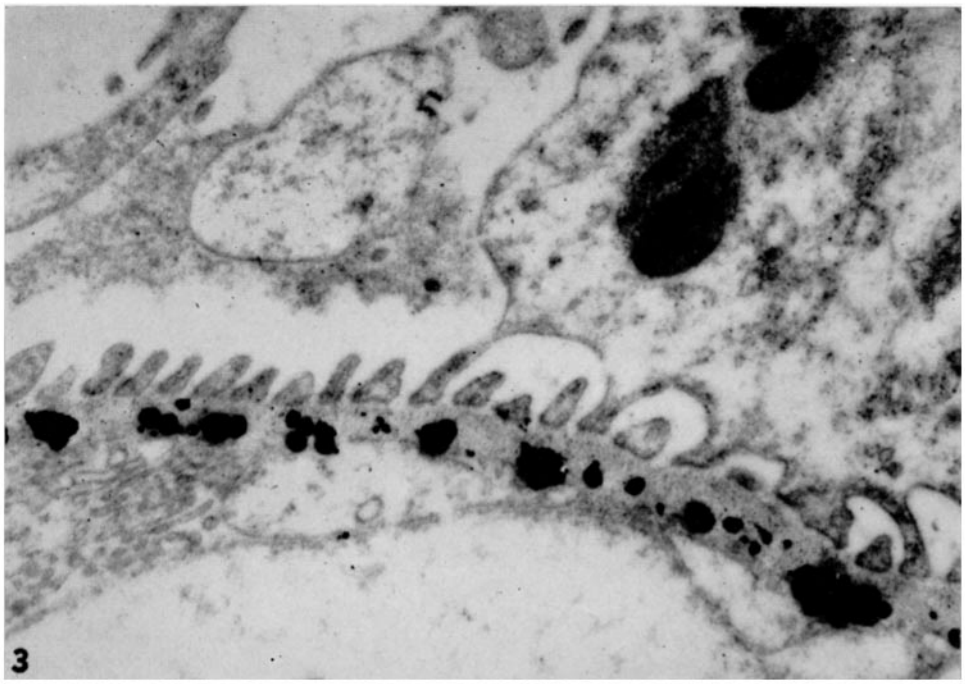


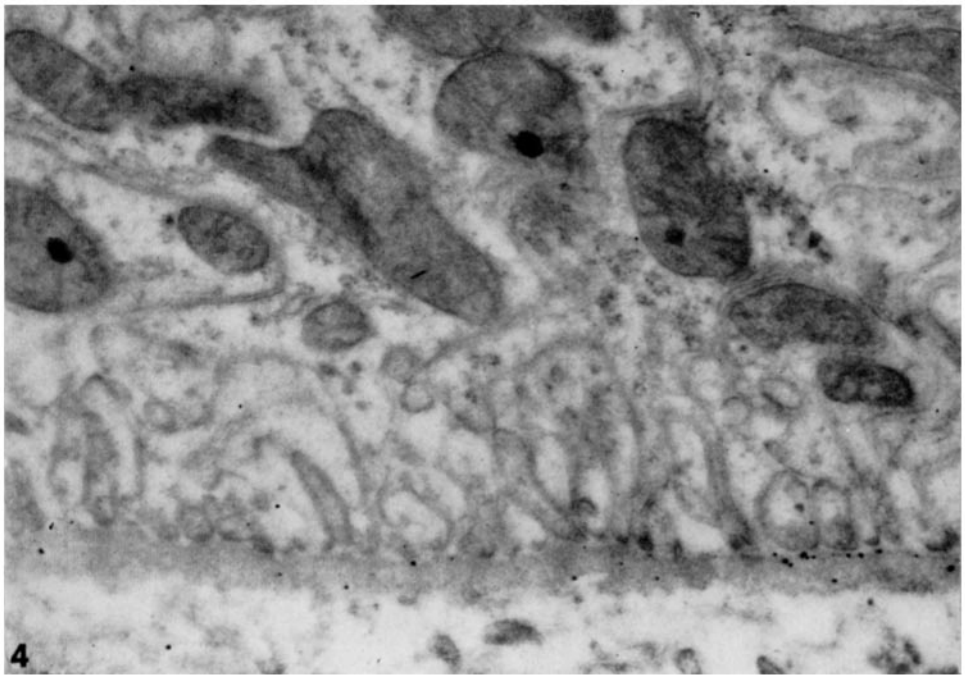
(Dempsey and Wislocki: Electron microscopy of argyria)

PLATE 32

FIG. 3. Section through a glomerular capillary and its associated pericyte from a mouse given silver for 6 months. The capillary lumen is at the bottom of the figure. Just above the endothelium is the capillary basement membrane, which contains silver granules ranging in size down to the limits of the electron microscope. At the top of the picture are portions of pericytes with end-feet resting upon the basement membrane. The pericyte at the right contains some silver deposits in spheroidal structures located toward the top of the picture. × 50,000.

FIG. 4. Portion of the base of a renal proximal convoluted tubule from a guinea pig which had received silver for 6 weeks. Granules can be seen in the mitochondria and in the basement membrane but not in the extracellular channels created by the recurving plasma membrane. × 25,000.



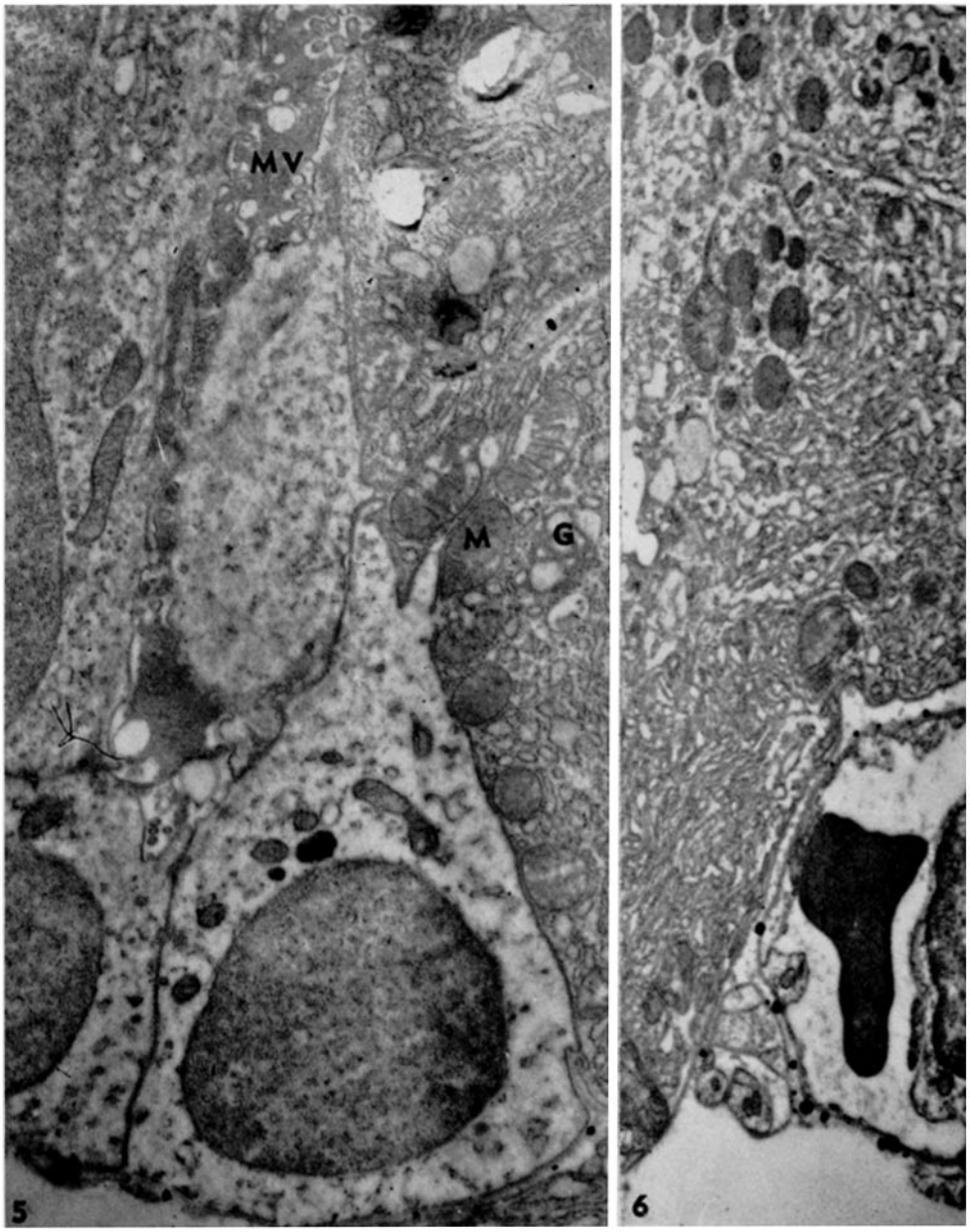


(Dempsey and Wislocki: Electron microscopy of argyria)

PLATE 33

FIG. 5. Section through the pancreas of a rat which had received $AgNO_3$ in its drinking water for 10 months. Running from the top center downward is the lumen of an acinus passing into the lumen of an intralobular duct. The apices of two acinar cells are seen along the right margin of the picture. Deposits of silver occur in the basement membrane of the duct (bottom of picture) and in bodies which resemble mitochondria located in one of the duct cells. Mitochondria (*M*), the Golgi region (*G*), microvilli projecting into the lumen of the duct (*MV*), and zymogen granules (*Z*) are illustrated. The granular outlines of the slightly dilated ergastoplasmic sacs can be seen in the acinar cells. × 15,000.

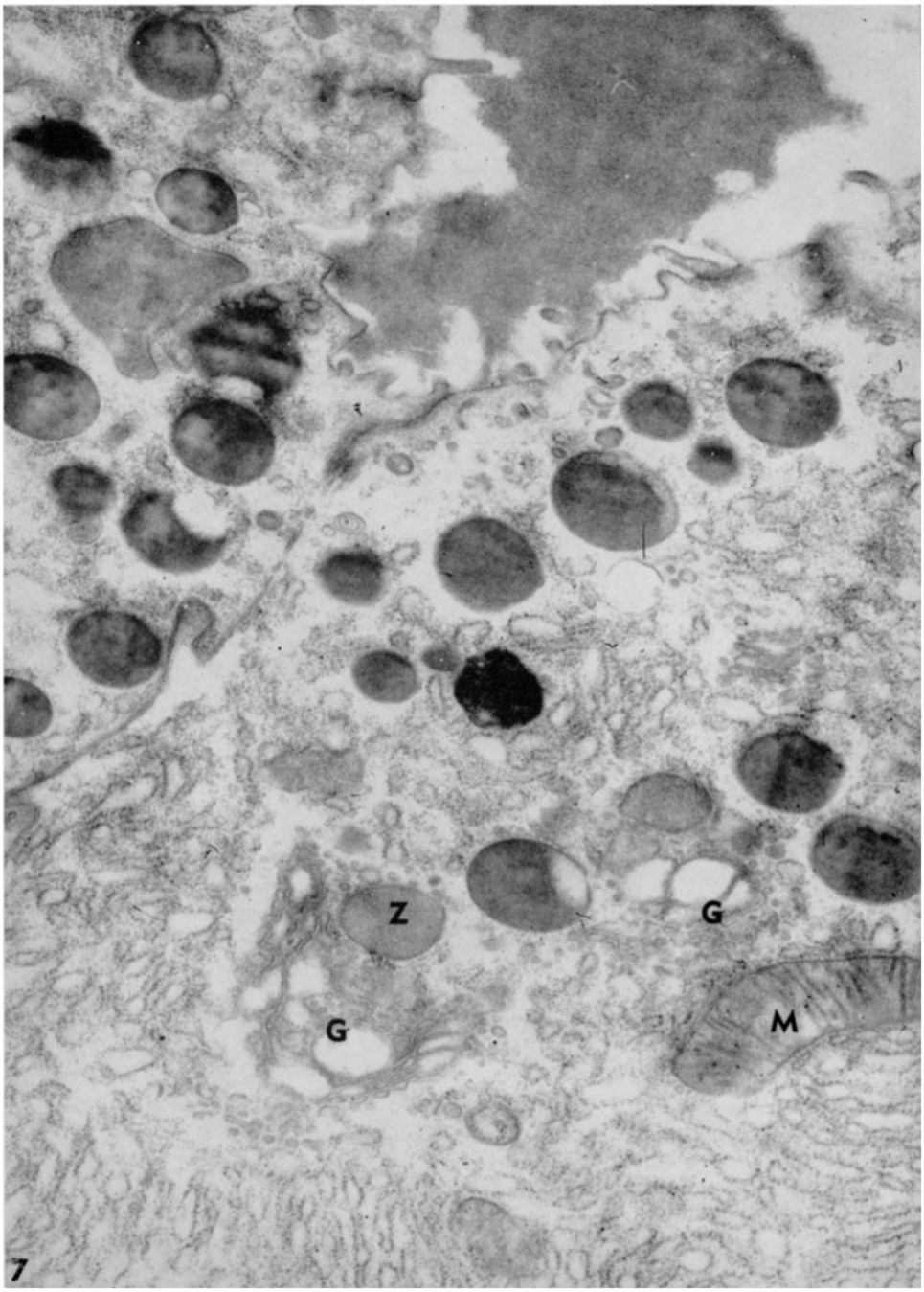
FIG. 6. Section through the base of an acinar cell from the same pancreas as illustrated in Fig. 5. A capillary with a single erythrocyte is shown at the bottom right. Several silver granules can be seen in the basement membrane of the endothelium. × 10,000.



(Dempsey and Wislocki: Electron microscopy of argyria)

PLATE 34

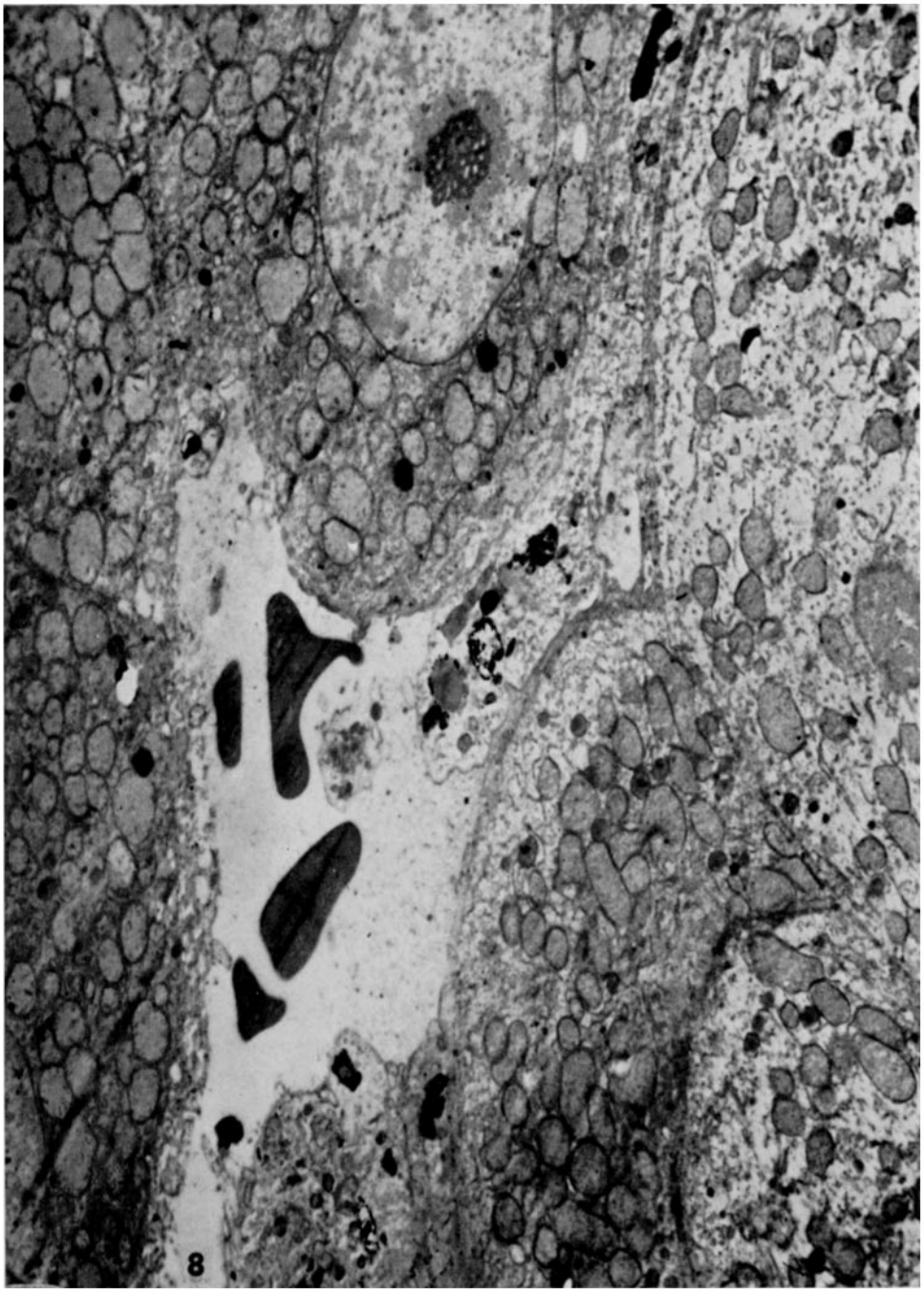
FIG. 7. Section through the apical parts of two acinar cells from the rat utilized for Figs. 5 and 6. At the top right is the acinar lumen, with microvilli projecting into it. Several dark, spherical zymogen granules (*Z*) can be seen. A few mitochondria (*M*) with their internal folds occur in the lower cell. At the lower center, two regions with small tubules and vesicles associated with larger spherical spaces exhibit the appearance Dalton has ascribed to the Golgi apparatus (*G*). At the center of the figure a spheroidal accumulation of silver granules can be seen. × 30,000.



(Dempsey and Wislocki: Electron microscopy of argyria)

PLATE 35

FIG. 8. Section through hepatic sinusoid and hepatic cells from a rat fed silver for 10 months. Projecting into the sinusoid are portions of three Kupffer cells, each of which contains vacuoles filled with silver granules. Dark granules can be seen in some of the hepatic mitochondria. × 5,000.



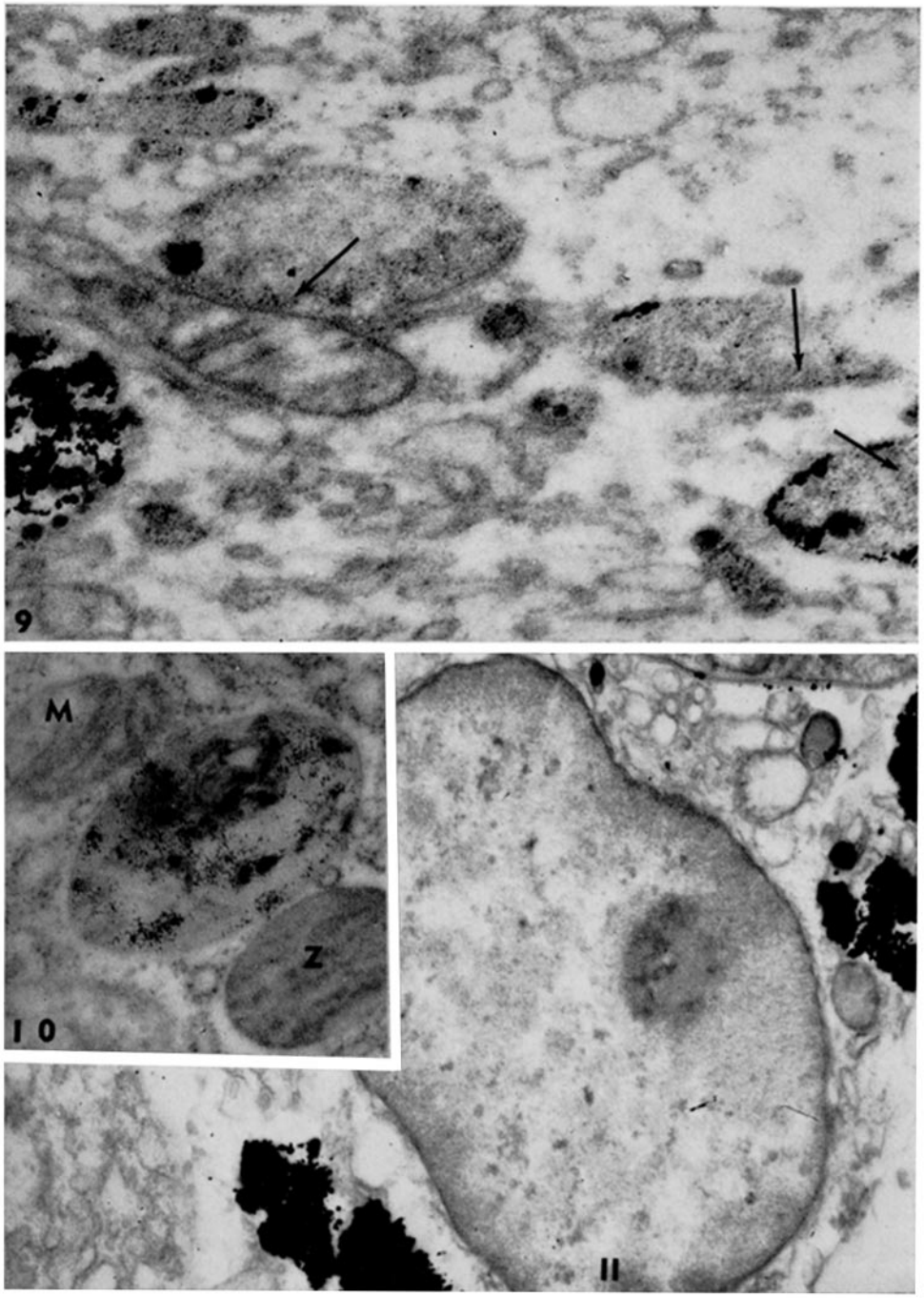
(Dempsey and Wislocki: Electron microscopy of argyria)

PLATE 36

FIG. 9. An area from an hepatic cell from a rat given silver for 10 months. Two large structures containing considerable amounts of silver can be seen at the extreme left and right An elongated mitochondrion with no silver occupies the left center of the picture. Above it, and to the right are structures containing slight silver deposits in which the double outer membrane and fragmentary internal folds (arrows) characteristic of mitochondria can be seen. Smaller structures containing silver and other matrix material are scattered throughout the field. × 25,000.

FIG. 10. Section through the pancreas of a rat given silver nitrate for 10 months. A mitochondrion (*M*) is shown at the upper left, a zymogen granule (*Z*) at the lower right. In the center is a spheroidal body containing granules of silver and a clumped mass of membranous material which resembles the internal folds of mitochondria. × 30,000.

FIG. 11. Section through the pancreas of a rat given silver nitrate for 10 months. The edge of an acinus appears at the extreme top right, and exhibits some small silver granules located in its basement membrane. The rest of the figure illustrates a macrophage located in the interacinar connective tissue space. It contains large segregation vacuoles filled with granules of silver. × 15,000.



(Dempsey and Wislocki: Electron microscopy of argyria)