ELECTRON MICROSCOPY OF RENAL COAGULATIVE NECROSIS DUE TO dl-SERINE, WITH SPECIAL REFERENCE TO MITOCHONDRIAL PYKNOSIS

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Fishman and Artom [1] discovered the nephrotoxic action of dl-serine (alpha-amino beta-hydroxypropionic acid) in the course of a study concerned with the influence of dietary factors on tissue phospholipids in rats. Morehead, Fishman and Artom [2,3] and Wachstein [4] investigated the renal injury in greater detail. The lesion was regularly reproducible in male animals, with an incidence of 100 per cent. Following the intraperitoneal injection of 100 mg. of dl-serine, extensive renal necrosis occurred within several hours. The damage was found to be localized to the terminal portions of the proximal convoluted tubules, as evidenced by microdissection of such kidneys.[4] The injury is caused by the unnatural d-isomer; l-serine is completely innocuous.[5] Since the lesion apparently represented a pure form of coagulation necrosis, it seemed worth while to subject it to electron microscopic analysis.

MATERIAL AND METHODS

Young male rats of the Wistar strain, weighing approximately 150 gm., were used, and 100 mg. of dl-serine dissolved in a few cc. of distilled water were injected intraperitoneally or intravenously. Control rats received equi-molecular amounts of sodium chloride. Four animals each were sacrificed 1, 3, 6 and 24 hours following intraperitoneal injection and 15, 30 and 60 minutes after intravenous injection. Food and water were not withheld during the experimental period.

At sacrifice, small tissue blocks from one kidney were fixed in buffered osmium tetroxide and, after dehydration, embedded in epoxy resin. Ultrathin sections were stained for 60 minutes at 45° C., with 4 per cent uranyl acetate, and examined with an RCA electron microscope, EMU-3F. Thick sections (1μ) were stained with toluidine blue,[6] and viewed with the light microscope or examined unstained with a phase microscope. Sections from the second kidney were fixed in 10 per cent formalin and processed for conventional light microscopy.

RESULTS

Control Animals

For the purpose of comparison, a brief summary of the findings in control animals is appropriate. Since the lesions occurred mainly in

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the proximal convolutions, discussion will be limited to these structures. Our observations were in complete agreement with those of others.[7-9] The proximal portions of the proximal convoluted tubules have a more complex structure than the more distal parts. With the fixatives used in this investigation, the convoluted tubules had a closed lumen and the microvilli of the brush border were in close apposition. Under ideal conditions of tissue preparation, however, the lumen of the tubules is apparently open.[10] The apical portions of the cytoplasm, beneath the brush border, contained many vesicular and membranous profiles (Fig. 1). The nucleus was located in the mid portion of the cell. This region also contained many vesicular bodies which were round or oval-shaped, and measured from 0.5 to 1 μ in diameter. They were lined by single smooth membranes and often contained fragments of dense filamentous material. These structures were considered to be cytosomes. Other similar organelles contained electron-dense "nucleoid" bodies. Only occasionally there were single membrane-limited vesicular bodies, measuring 0.1 to 0.2 μ, containing a number of small vesicles. Mitochondria were numerous and exhibited the well-known perpendicular arrangement. They were located within cytoplasmic compartments formed by infolding membranes (beta-cytomembranes) which reached deeply into the cytoplasm. The Golgi apparatus was not prominent. Endoplasmic reticulum formed slender tubules and vesicles. The more distal portions of the proximal convoluted tubules showed a simpler organization. The microvilli were not as regular, and the infolding membranes were shallower. The mitochondria were less numerous and were more randomly distributed.

Experimental Animals

As early as 15 minutes after intravenous injections of dl-serine, definite focal lesions could be detected electron microscopically. In animals sacrificed 30 and 60 minutes after intravenous, and 3 and 6 hours after intraperitoneal injection, these increased in extent and severity. In the kidneys of rats sacrificed 24 hours later, the cells of most of the affected tubules had become completely necrotic.

In sections cut at 1 μ and treated with toluidine blue in animals sacrificed within the first 6 hours, there were intensely stained foci of coagulative necrosis in individual tubular cells. The neighboring cytoplasm in the same cell was normal initially or more loosely textured and less intensely stained. As the lesion progressed, diffuse density spread through the cytoplasm and extended into the brush border. Small vacuoles, mostly in noncoagulated portions of the cytoplasm, were evident.

With the electron microscope, two main changes were distinguished; these corresponded to those observed in thin sections with the light microscope. They have been designated "vacuolar" and "coagulative" alterations (Fig. 2). The latter was characterized by increased generalized density of the cytoplasm, the former by increased vacuolation. In earlier phases of the injury, a varying degree of rarefaction of the cytoplasmic matrix with disruption of the orderly arrangement of smooth and rough endoplasmic reticulum were encountered (Fig. 3). In most fields, however, marked vesiculation of the cytoplasm was also noted (Figs. 2 and 4). Occasionally the inner surface of the limiting membrane of such vesicles had a smudgy outline. The microvilli in the brush border often showed striking vacuolation not only near their cytoplasmic origin, but also close to their tips (Figs. 2 and 5). At a more advanced stage of cellular damage, the microvilli became fragmented and disintegrated, forming small vesicles (Fig. 6).

Structures resembling multivesicular bodies were very frequent (Figs. 5 to 7). These varied in size from 0.1 to about 2 μ in diameter and were always surrounded by single membranes. In areas in which the cytoplasm contained many tiny vesicles, multivesicular bodies were more frequent and occasionally lacked limiting membranes (Fig. 6). There was also an increase in the number of cytosomes and of the dense bodies (Figs. 5 and 8).

There was complete lack of fluid retention between infolding membranes in the extracellular compartments. On the other hand, rupture and dissociation of these membranes was a frequent occurrence (Fig. 3). Typical myelin figures appeared in completely necrotic cells, but were not numerous. More common were accumulations of tubular and vesicular structures forming round bodies which measured up to 3 μ in diameter (Fig. 3). The Golgi apparatus exhibited no significant alterations (Fig. 8).

The mitochondria underwent two kinds of changes, resulting in either shrinkage or swelling of these organelles. Swollen mitochondria often had irregular outlines (Fig. 11). The cristae exhibited disappearance of their centrally located portions; short segments attached to the inner mitochondrial membranes remained as closed tubules. Occasionally the mitochondria were transformed into cysts lined, in the main, by double membranes.

Shrinkage led to a striking increase in the density of the mitochondrial matrix which was associated with an over-all reduction of mitochondrial size (Figs. 3 to 5, 8, 10 and 12). In general, the shape of the dense mitochondria was not altered. The spaces between two juxtaposed cristae were often more distinct than in normal organelles. We

have designated this mitochondrial alteration "pyknosis." Occasionally several such pyknotic mitochondria were fused together (Fig. 9), and the conglomerates were often surrounded by a single membrane.

Coagulative cytoplasmic changes were less complex than the vacuolar ones. Initially small foci of cytoplasmic condensation within single cells were noted. These spread through the entire cell and extended into the brush border (Fig. 2). Occasionally, the infolded plasma membranes acted as barriers against the further extension of the process.

Coagulative necrosis apparently occurred in cells otherwise unchanged, or appeared in areas which had developed a vacuolar structure. The coagulative foci were characterized by increased electron density of all cytoplasmic components, and their finer details were obscured on occasion. Whenever coagulation extended to the lumen border, the microvilli appeared uniformly dense and became separated from the cytoplasm, subsequently undergoing fragmentation into several portions. The nuclei were shrunken and exhibited serrated, irregular outlines.

In rats sacrificed 3 or more hours following the administration of dl-serine, the necrotic epithelium became separated from the underlying basal membrane (Fig. 12). Necrotic cells broke into clumps which appeared in the lumen. Nuclei here were completely necrotic.

The remaining portions of the nephron showed no consistent changes, with the exception of an apparent increase in the number of vacuoles in the ascending limbs of Henle's loop.

Discussion

The process of cellular necrosis has been investigated by histologic and histochemical techniques.[11–13] Recently, ultrastructural features have also been reported.[14–17] The lesion induced by dl-serine was considered of pertinence because coagulation necrosis occurred reproducibly and regularly within a short period.

In thick sections cut at 1 μ, two cytoplasmic changes were noted, and these could be scrutinized in greater detail with the electron microscope. The alterations were considered "coagulative" and "vacuolar."

Vacuolar Changes

Prominent vesicles and vacuoles imparted a rarefied appearance to the cytoplasm. Initially, areas of decreased cytoplasmic density were unaccompanied by vacuolation. In such areas, RNP particles were disarrayed and clumped together in irregular aggregates; later they disappeared completely. A similar loss of cytoplasmic electron density has also been described in other experimental conditions.[18,19] In most af-

fected cells, however, the cytoplasm contained many vacuoles surrounded by single membranes. The vacuoles might have been derived from distended smooth and rough endoplasmic reticulum.[20–25] Small vacuoles could also have originated in the brush border and in cystically dilated mitochondria.[22,26,27]

Two other structures could possibly have contributed to the cytoplasmic vacuolation; these, however, were apparently not involved. The Golgi apparatus showed no significant changes. Infolded plasma membranes were not separated from each other, and there was no enlargement of the extracellular fluid compartments between the infolded membranes.[28–31]

Not all vacuoles in the abnormal cells necessarily originated in preformed ultramicroscopic structures. In parenchymal liver cells of rats subjected to hypoxia, increased vacuolation noted electron microscopically [23,32–34] could have been due to increased pinocytosis.[35] Vacuolation attributable to pinocytosis is readily observed in normal renal tubular cells at the height of diuresis.[30] Thus it is possible that cellular damage stimulates physiologic processes. With impending cell death, however, the process of pinocytosis comes to an end.[36] Other factors may also lead to cellular vacuolation. In injured cells a rise of osmotic pressure, probably due to accumulation of intermediary metabolites, may lead to influx of fluid into the cell. An increase in the permeability of cell membranes may have a similar result.

Cells with increased cytoplasmic vacuolation exhibit a number of additional features. Three types of single membrane-limited bodies appear in normal rat renal epithelium: cytosomes, microbodies and multivesicular bodies. In rats given serine injections, the latter exhibit an impressive increase, not only in number, but also in size. Multivesicular bodies are found in many organs [37] and have been shown to gather colloidal particles in cells of the glomerulus in normal and nephrotic rats.[38] It seems probable that in the serine-induced injury, the formation of these bodies was initiated by the abundance of newly formed small vesicles. Cytosomes as well as microbodies were also increased in number.

If one assumes that cytosomes are concerned with the disposal of foreign proteins,[39,40] one may infer that in damaged cells these structures can be associated with the disposal of endogenous cellular debris as well. Obviously, cytosomes are not stable cytoplasmic constituents, but increase in response to increased supply of disposable material. Cytosomes contain acid phosphatase as well as other hydrolytic enzymes, and have been identified with de Duve's lysosomes.[37] More recently, cytoplasmic structures have been recognized by electron microscopy which are also limited by single membranes, and which may

contain a variety of organelles such as mitochondria, vacuoles or endoplasmic reticulum.[41–43] Such areas also exhibit acid phosphatase activity.[44] They represent foci of cytoplasmic degradation and can occur in various unrelated abnormal conditions, and even in apparently normal cells. In contrast to the increase in number of cytosomes, focal cytoplasmic degradation was not too frequent in the serine-damaged cells.

Further evidence of cellular disturbances was indicated by the break-up of infolded beta-cytomembranes, and the appearance of peculiar tubular and vacuolar structures resembling hypertrophied smooth endoplasmic reticulum. Hypertrophy of endoplasmic reticulum has been described in the liver of rats two days after the administration of 3′-Me-DAB,[24] within 20 hours after dimethylnitrosamine,[25] and in the kidneys 14 hours after the administration of Kollidon (polyvinylpyrrolidone).[18] Lipophanerosis,[45] which is responsible for the formation of myelin figures, occurs in a variety of injurious states and may contribute to the new formation of these tubules and vacuolar structures.

Mitochondria exhibit two types of alteration: swelling and shrinkage. Swelling of mitochondria was encountered early in serine injury, and even then involved only part of the mitochondrial population. Swelling of these organelles is a feature of many abnormal states; [14,20,27,32,33,35,46,47] indeed, it may even occur in starvation.[48 49] In all these conditions, swelling is associated with loss of mitochondrial matrix and at least partial disappearance of cristae. The process apparently differs from that in mice on diets deficient in essential fatty acids. In these, mitochondrial swelling is associated with an increase in the number of cristae.[50]

The usual mitochondrial alteration observed consisted of a striking increase in the density of the matrix and a reduction in mitochondrial size. We have termed this phenomenon "pyknosis." To our best knowledge, mitochondrial pyknosis has been infrequently reported. It has been observed in the liver of rats re-fed after a period of starvation or given cancerogenic diets.[51,52] It has also been noted in hepatomas [48] and in experimentally induced renal tumors in hamsters.[53] Marked increase in the density of mitochondria also occurs in the liver of rats on a necrogenic yeast diet, even before the onset of necrosis.[54]

If appropriately prepared tissue sections are digested with ribonuclease, the ergastoplasm disappears while the matrix of the mitochondria increases considerably in density.[55] Electron micrographs of liver mitochondria isolated in hypertonic sucrose solution, as illustrated by Rouiller,[26] show organelles strikingly similar to the pyknotic mitochondria in serine-injured cells. Occasional mitochondria are not

shrunken but swollen. Thus mitochondrial shrinkage *in vivo* and *in vitro* cannot be attributed to variations in osmotic equilibrium alone.

Coagulative Changes

Diffuse coagulative changes are characterized by a uniform increase in electron density in which all submicroscopic structures participate. This process begins in limited areas of cells which may or may not exhibit pre-existing vacuolation. The increased cytoplasmic density is obviously due to loss of fluid. Areas of coagulative necrosis are sometimes limited to single cellular compartments delineated by infolding beta-cytomembranes.[18] More often, however, the necrosis begins in a focus close to the basement membrane and extends through the entire cell. Within these areas and occasionally also in vacuolated portions of the cytoplasm conglomerates of fused, dense mitochondria were encountered. Similar aggregates were described in the liver of rats given diethylnitrosamine.[56] It is unlikely that these accumulations represent evidence of mitochondrial proliferation, as in the livers of rats on a necrogenic diet in the prenecrotic phase,[54] or in the livers of African monkeys given injections of yellow fever virus.[57] In these situations, mitochondria appear otherwise normal and not shrunken.

It is obvious that cytoplasmic vacuolation can be caused by a number of different changes in various subcellular structures. These alterations are reversible and the cell may survive after the harmful situation has been corrected. Thus, if the blood supply is re-established in experimentally induced liver anoxia, the striking hepatic cell vacuolation subsides completely.[23] With the onset of mitochondrial pyknosis or uniform coagulative necrosis, however, the injury becomes irreversible.

Summary

In the regularly induced coagulation necrosis occurring in the distal portion of the proximal renal tubule convolution in rats after administration of dl-serine, two types of cytoplasmic lesions were distinguished. These were "vacuolar" and "coagulative" and often co-existed in the same cell. The vacuolar lesion appeared in cytoplasmic matrix with lessened electron density and was most frequently characterized by an increased number of vesicles. These could have originated from distended endoplasmic reticulum, in vacuolated, fragmented brush border microvilli or from swollen mitochondria. There were also conglomerations of tubular and vesicular structures, possibly originating from the endoplasmic reticulum. Cytosomes and particularly multivesicular bodies were markedly increased in number and size. The majority of

mitochondria appeared shrunken and exhibited increased matrix density.

In cytoplasmic coagulative necrosis, increased density was observed not only in mitochondria but in all other subcellular structures as well.

It was assumed that vacuolar changes, which may occur in a variety of states (e.g., anoxia) indicated a moderate degree of reversible cellular damage. The onset of mitochondrial pyknosis and cytoplasmic coagulative necrosis, however, signified an irreversible lesion.

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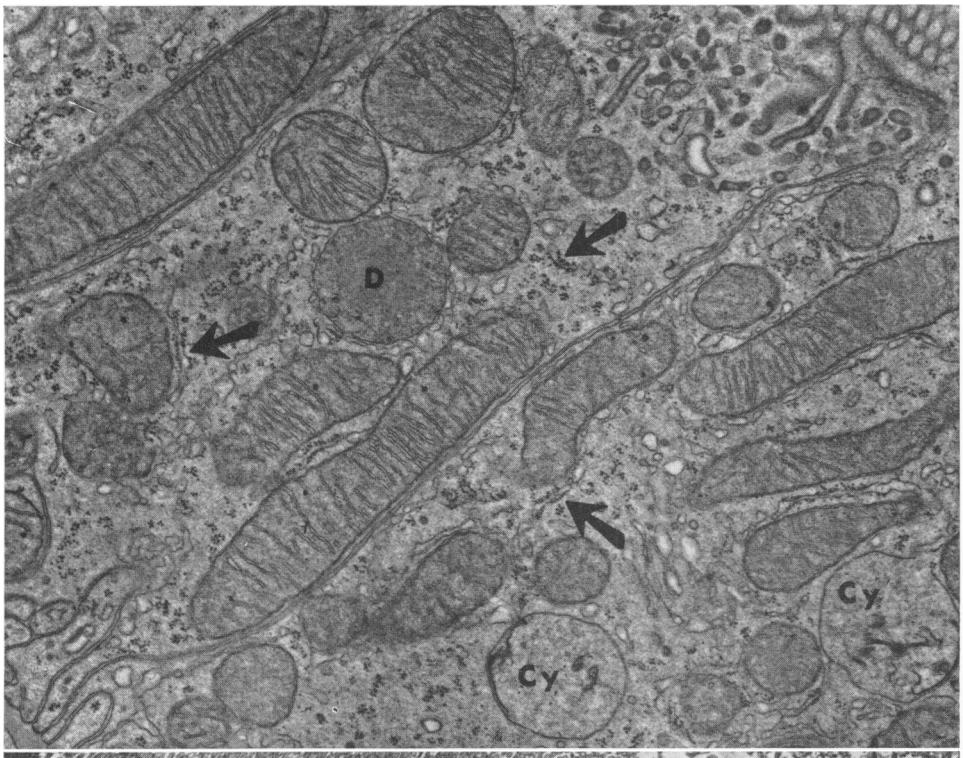
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. A portion of a normal cell in the proximal tubular convolution of a rat kidney. In the left lower corner, basement membranes and infolded plasma cellular membranes appear. In the right upper corner, cross sections of microvilli of brush border are evident. Beneath the brush border there are small vacuolar and tubular structures. Apart from elongated mitochondria, two cytosomes (Cy) and one dense body (D) are seen. RNP particles are visible throughout the cytoplasm. Arrows, endoplasmic reticulum. × 16,400.

FIG. 2. Three hours after the intraperitoneal injection of dl-serine. Extensive coagulation necrosis has occurred in several cells and vacuolar changes appear in a cell located to the left of the center. There is, however, pyknosis of mitochondria even in the vacuolated area. Tips of microvilli in the central upper part of the micrograph exhibit vacuolation. Microvilli overlying the diffusely pyknotic area to the right show condensation. × 5,780.



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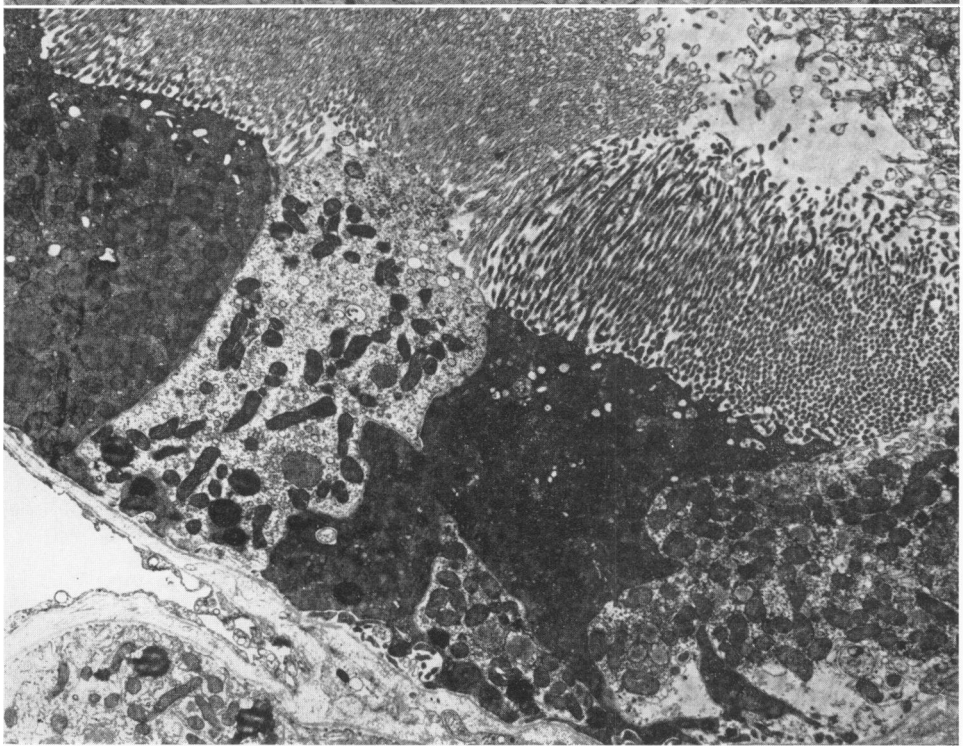

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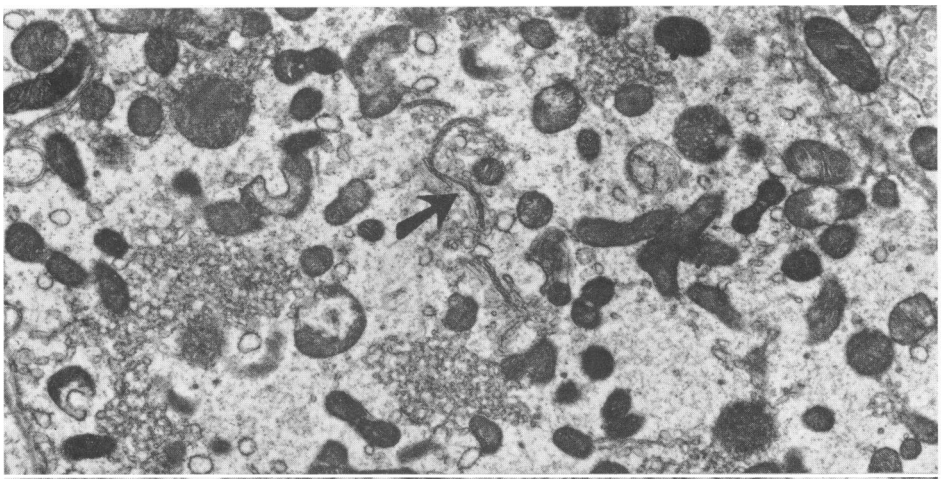
FIG. 3. There is rarefaction of the cytoplasm one hour after the intravenous injection of dl-serine. Large networks of tubular and vesicular structures are manifest. An arrow points to a fragment of infolded membrane. Mitochondria exhibit increased density. × 9,600.

FIG. 4. There is marked vacuolation of the cytoplasm. Mitochondria are pyknotic. × 21,400.

FIG. 5. One hour after the intravenous injection of dl-serine. There is marked vacuolation of the brush border. Arrows point to cytosomes which are increased in number. Multivesicular bodies are also evident; mitochondria are dense. × 9,600.



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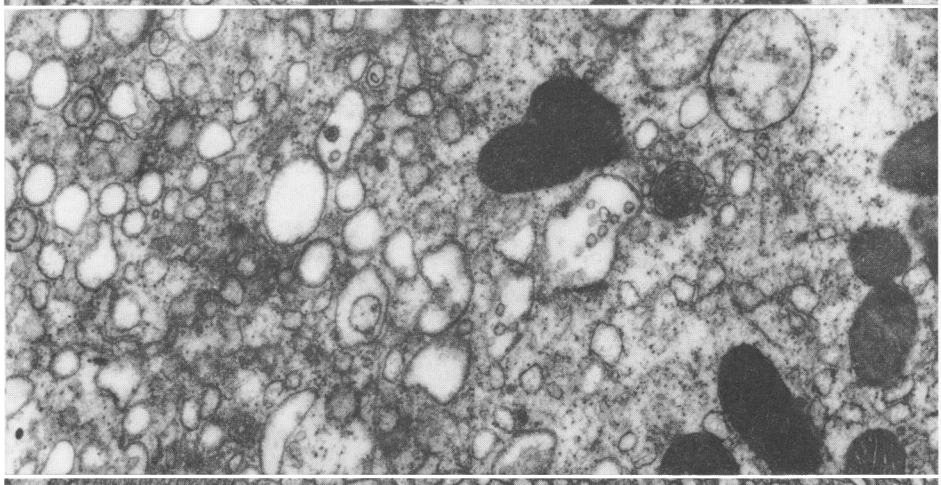

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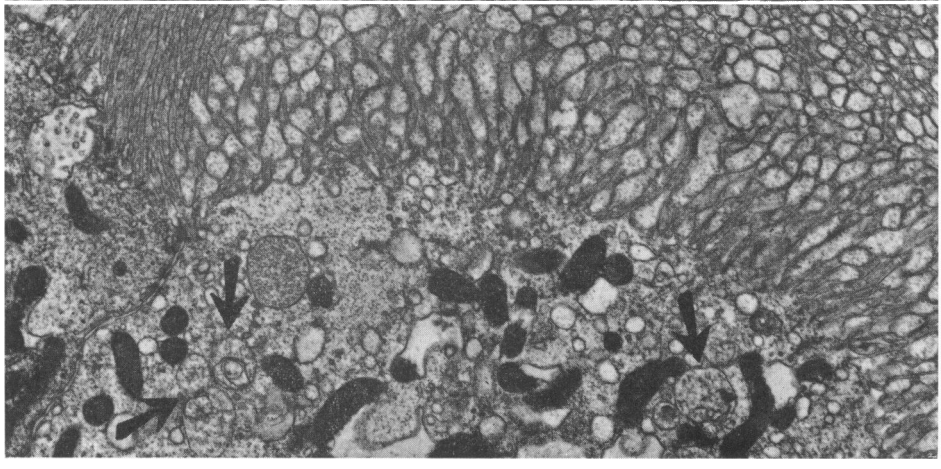


5

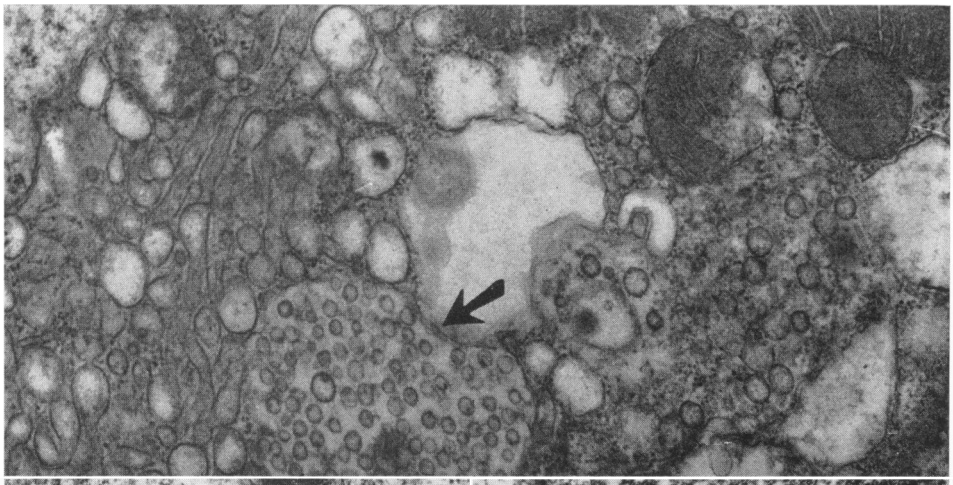
FIG. 6. To the left, vesicle formation is noted in microvilli. An arrow points to a collection of small vesicles which is only partially surrounded by a single membrane, suggesting the formation of a multivesicular body. × 24,600.

FIG. 7. A large multivesicular body. A smaller structure containing two larger and several small vesicles is also shown. × 25,200.

FIG. 8. There is marked increase in the number of cytosomes. Arrow points to the Golgi apparatus which appears essentially normal. × 15,000.

FIG. 9. Aggregates of fused pyknotic mitochondria are surrounded by single membranes. × 25,200.

FIG. 10. Higher magnification of pyknotic mitochondria. The mitochondrial matrix is dense and the spaces between two cristae are very prominent. × 31,200.



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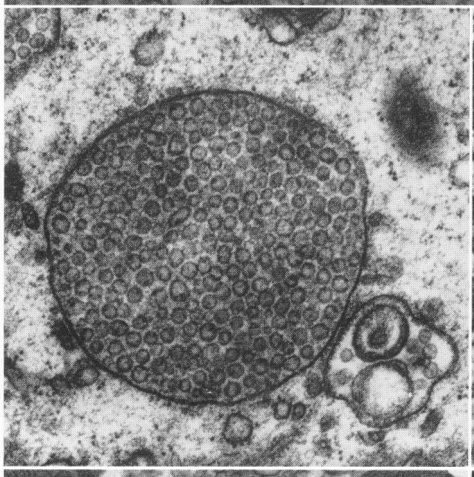

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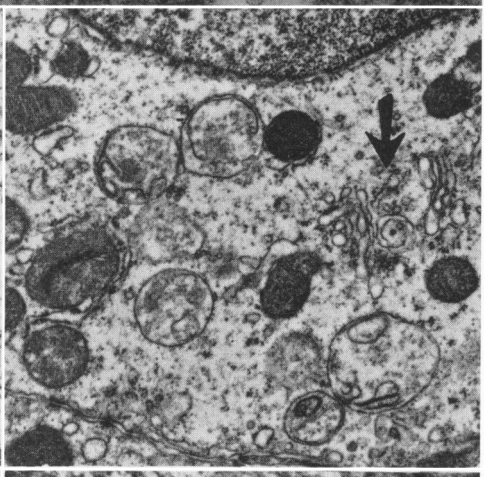

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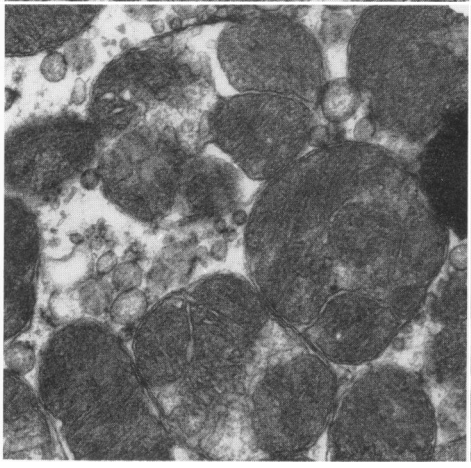

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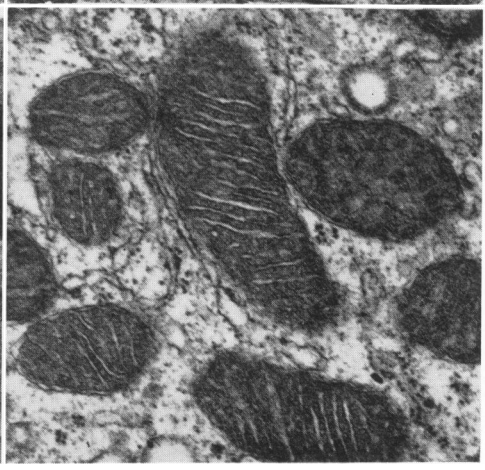

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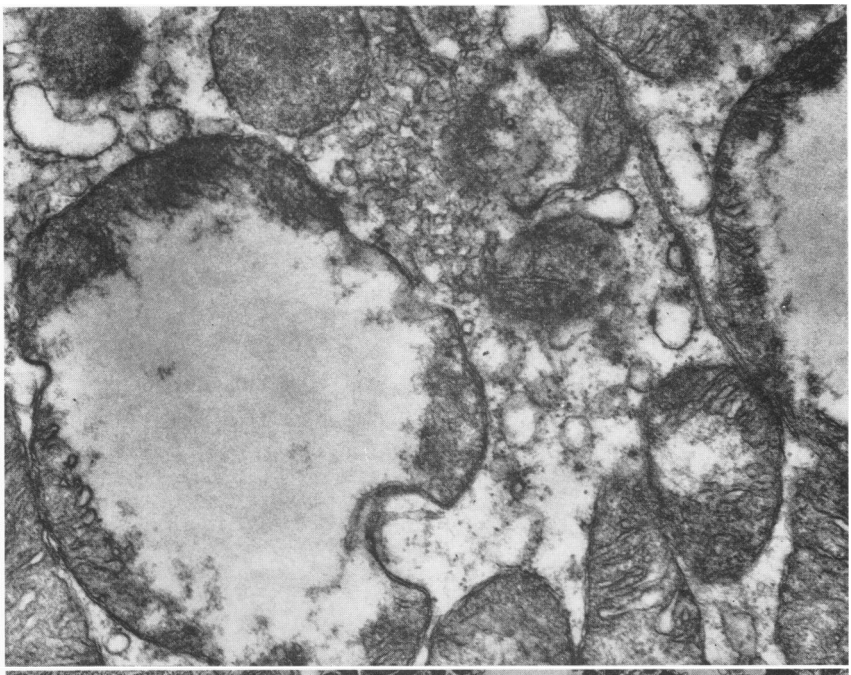

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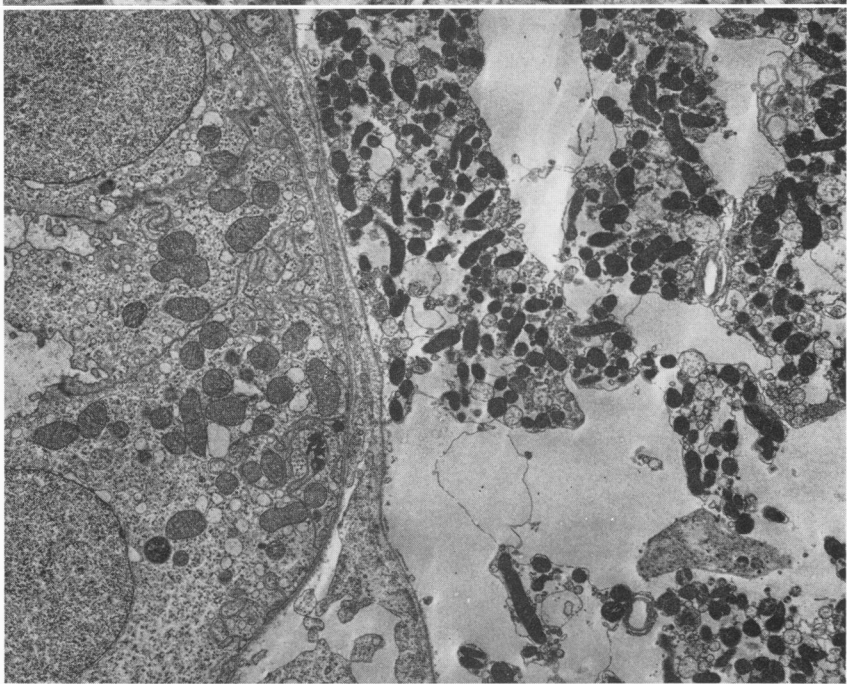

FIG. 11. Two markedly swollen mitochondria appear. Only remnants of cristae are attached to the inner mitochondrial membrane. × 26,000.

FIG. 12. Six hours after dl-serine injection there is complete tubular necrosis with denudation of the basement membrane and fragmentation of cells in the tubule to the right. Compare the density and shrinkage of mitochondria in the necrotic tubule with the normal appearing mitochondria in an undamaged collecting duct to the left. × 5,100.