MERCURY NEPHROTOXICITY IN THE RAT

2. Investigation of the Intracellular Site of Mercury Nephrotoxicity by Correlated Serial Time Histologic and Histoenzymatic Studies

A. E. Rodin, M.D., M.Sc. (Med.), F.R.C.P.(C),*
and C. N. Crowson, M.A., M.D., Ph.D.†

Winnipeg, Manitoba, Canada

Mercury has been recognized as a nephrotoxic agent for several centuries. The fact that renal disorder could be produced by mercurial salts used in the treatment of syphilis has been known since the 16th century.[1] Overbeck, in 1860,[2] regarded the presence of protein in the urine during the course of treatment with mercurial substance to be due to a "simple catarrh of the kidney." In the same year Taylor[3] reported the case of the woman who had been acquitted by English courts of murdering her husband with white precipitate (79 per cent mercury); the consensus of medical opinion at that time held that mercury was not a poison. To dispute this, Pavy[4] investigated the effect of orally administered white precipitate on dogs, rabbits and mice; he found gross renal alterations consisting of enlarged, roughly speckled kidneys with marked striation of the cortex.

Moore, Goldstein and Canowitz, in 1929,[5] examined the mitochondria of renal tubular epithelium following mercury administration histologically and found that definite degenerative changes occurred in the cytoplasm and nucleus before any qualitative alterations occurred in the mitochondria. Therefore, they suggested that mercury bichloride produced nephrosis by adsorption on the surface of mitochondria, "poisoning" the interface and later killing the cell. Oliver, in 1932,[6] also noted mitochondrial agglutination. Oliver, MacDowell and Tracy, in 1951,[7] studied kidneys in mercury bichloride poisoning and found focal lesions in the proximal convoluted tubules. This they termed nephrotoxic nephrosis and attributed the lesion to the direct toxicity of mercury on tubular epithelium. Other investigators, in recent times, have localized

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* Present address: Department of Laboratory Medicine, Misericordia Hospital, Edmonton, Alberta, Canada.

† Present address: Director of Laboratories, Misericordia Hospital, Winnipeg 1, Manitoba, Canada.

the mercury nephrotoxic lesion to the more terminal portions of the proximal convoluted tubules.[8]

Investigations of the histoenzymatic changes in nephrotoxicity have been concentrated on oxidation-reduction enzymes, particularly succinic dehydrogenase. A study of oxidation-reduction enzymes has the advantage that, unlike alkaline phosphatases,[9] their activity is not limited to the proximal convoluted tubules but is present in varying degrees of intensity throughout the nephron.[10] Succinic dehydrogenase is located within the mitochondria of most living cells[11] and is present in greatest concentration within the physiologically and biochemically active organs such as heart, kidney and liver.[10] A component of the Krebs cycle, the enzyme is concerned with the oxidation of succinate to fumarate, cytochrome C acting as the hydrogen acceptor. Determination of succinic dehydrogenase activity in animal tissues has been accomplished by estimating the rate of oxygen uptake in the presence of succinate and cytochrome C[12] and by utilization of electron acceptors such as methylene blue, brilliant cresyl blue, tellurites and tetrazolium.[13] Tetrazolium salts are the most sensitive indicators of succinic dehydrogenase activity,[14] the tetrazolium being reduced to a black formazan pigment.

As mercury inhibits sulfhydryl groups by forming mercaptides,[15] it has been suggested that mercury-induced diuresis is produced by inhibition of the succinic dehydrogenase enzymatic system by combination of mercury with the -SH groups.[16] The development of tetrazolium stains for demonstrating succinic dehydrogenase in tissues[17] led to the investigation of the site of renal mercurial diuretic action. Mustakallio and Telkkä[18] found that following injections of mercurophylline in rats, succinic dehydrogenase activity disappeared from the proximal convoluted tubules and the thicker portions of Henle's loops. Wachstein and Meisel[19] found a decrease in succinic dehydrogenase activity in the terminal portion of the proximal convoluted tubules within 45 to 60 minutes after the injection of a toxic dose of a mercury compound. If mercury-induced renal tubular lesions show a reduction of dehydrogenase activity before the onset of histologic changes, as suggested by Wachstein and Meisel,[20] either ischemia or "poisoning" of the Krebs cycle may be implicated in mercury nephrotoxicity. If a decrease in dehydrogenase activity does not precede histologic alteration, it is more likely secondary to cellular degeneration. The specific objective of the experimental series to be described is the serial time study of the onset of changes in succinic dehydrogenase activity and in histologic integrity of renal tubules in rats given mercury bichloride.

Method

Preliminary histochemical studies to assess normal rat kidneys were undertaken with 60 adult albino Wistar rats of both sexes. Similar rats (64) were utilized for

mercury bichloride injection studies. Eight of these were kept as controls, 1 for each time interval. The remaining 56 rats each received intramuscular injections of mercury bichloride at a dose of 0.005 mg. of mercury per gm. of body weight. This dosage was chosen because previous experimental work had shown that it produced a lesion limited to the terminal segment of the proximal convoluted tubule.[21] The 56 rats were then divided into 8 groups containing 7 animals each, these groups being sacrificed in turn at intervals of 1 hour, 2 hours, 3, 4, 5, 6, 9 and 12 hours.

The rats were given ether at the appointed time until respirations ceased. The kidneys were then quickly removed and bisected; one half of the kidney was used for conventional staining and the other half immediately processed for tetrazolium staining.

The incubating solution used for tetrazolium staining was patterned along the line of that first used by Seligman and Rutenburg.[17] However, instead of blue tetrazolium, a new nitrophenyl tetrazolium derivative (Nitro-BT) was used. Nitro-BT has the advantage that it is much more sensitive, requires a shorter incubation period and results in the deposition of dinitro-formazan which is not lipid-soluble.[22]

One half of the fresh kidney was placed on the freezing microtome immediately after removal, and sections were cut at 20 μ. Throughout the entire project two sections were made from each kidney. The sections were then dipped into physiologic saline, placed into individual glass cups containing 5 cc. of the incubating solution and incubated for 1 hour at 32° C. After incubation the sections were floated in normal saline, mounted on glass slides, fixed in 10 per cent formalin overnight and then rinsed in distilled water.

The sections were then placed into tetrahydrofuran solution for 6 minutes for the purposes of dehydrating and clearing. Tetrahydrofuran was found to have advantages over alcohol and benzine derivatives because shrinkage was reduced to a minimum[23] and artifactual formazan deposition between tubules and in perirenal fat was dissolved without affecting intracellular formazan. After dehydration and clearing, a drop of clarite was added to the slide and a cover slip applied.

Normal Rat Kidney

Observations

A study of the sections of the normal rat kidney stained with the Nitro-BT derivative of tetrazolium revealed an adequate degree of uniformity throughout the series. There were only slight differences in intensity among the sections obtained from different normal rats. Multiple sections made from the same kidneys showed identical staining. All sections had a consistent pattern of distribution of dinitro-formazan pigment deposition within individual tubular cells and in different parts of the nephron. The stained elements within epithelial cells were sufficiently well delineated to permit adequate photomicrographic representation.

Previous studies of normal rat kidneys stained conventionally showed that hemisections are composed of 4 zones.[21] There is an outer cortex containing glomeruli, distal convoluted tubules, loops of Henle and the juxtaglomerular and middle thirds of proximal convoluted tubules. The inner cortex is composed largely of the terminal third of proximal convoluted tubules and does not contain glomeruli. The outer medulla largely contains loops of Henle; the inner medulla is comprised of collecting tubules.

Examination of the tetrazolium-stained slides with the naked eye revealed 4 definite zones corresponding in size to the 4 zones observed with conventional staining (Fig. 1A). The first zone corresponded to the outer cortex and stained an intense bluish black with innumerable minute nonstained bodies representing glomeruli. The second zone corresponded to the inner cortex and had a less intense bluish coloration, with absence of unstained bodies. Although the first and second zones were not sharply demarcated from each other, the third zone was abruptly demarcated from the second, the junction being a straight line. This third zone, representing the outer medulla, had dark bluish black striations, but these were irregularly separated by clear, relatively nonstained striations. The stained striations represented the second portions of Henle's loops and the clear structures were the collecting tubules. The fourth zone, the inner medulla, was pale and clearly demarcated from the third zone by a straight line. Close inspection revealed faint, patchy areas of staining in the outer half of the inner medulla.

The upper two thirds of the proximal convoluted tubules, the loops of Henle and the distal convoluted tubules all showed an intense degree of bluish black staining. Although occasional cells had such intense staining as to appear structureless, the majority contained fine, regular, minute punctate dots. An occasional tubule in the outer cortex showed considerably less staining than the others because of a decreased concentration of punctate dots which were less intensely stained. These were the initial portions of collecting tubules. Nuclear areas could be distinguished in many cells and there were also demarcated oval to round areas of lesser staining. In the cells with intense stain reaction, nuclear areas were not as evident.

The inner cortex was somewhat paler staining than the outer because of less intense staining in the terminal thirds of the proximal convoluted tubules (Fig. 2). The staining pallor appeared to be due to both a decreased number of punctate dots and a lesser degree of reaction. Occasional cells showed more intense staining reaction, but this was limited to the region adjacent to the basement membrane. Also present in the inner cortex were intensely stained loops of Henle and poorly stained collecting tubules, both tending to run in a vertical manner. The tubules in the outer medulla lay in a vertical plane as well, and here, too, intensely stained loops of Henle were interspersed among the poorly stained collecting tubules.

In the inner medulla the collecting tubules retained a faint stain reactivity for a short distance from the outer medullary zone (Fig. 3). The proximal portions of collecting tubules also contained occasional small cells with staining intensity equal to that seen in the terminal por-

tions of the proximal convoluted tubules. The distal portions of the collecting tubules were completely lacking in formazan staining; this was also the case in the pelvic mucosa.

Discussion

The most intensely formazan-stained portion of the nephron was the epithelium in the upper two thirds of the proximal convoluted tubule, the loop of Henle, and the distal convoluted tubule. The terminal third of the proximal convoluted tubule stained less intensely, producing a pale zone between the outer cortex and outer medulla. Padykula,[10] Mustakallio and Telkkä,[24] and Wachstein and Meisel[25] have made similar observations. However, Pearson[26] and Nachlas, Tsou, de Souza, Cheng and Seligman[27] described no difference in staining in different segments of the proximal convoluted tubule, even though the latter investigators used the Nitro-BT method.

The collecting tubules exhibited slight activity in the proximal portion of the cortex, the outer medulla and the superficial layer of the inner medulla. Larger collecting tubules and ducts of Bellini were completely nonreactive. Of special interest were the scattered small cells with active formazan staining in the proximal portion of the collecting tubules. These were comparable to the dark cells described by Rhodin[28] in this region. With the electron microscope they contained many more mitochondria than companion cells in the collecting tubule.

A major consideration related to the identity of the formazan-stained intracellular units. The minuteness, sharpness and regular and even distribution of the blue-black formazan granules within the cytoplasm of tubular epithelium immediately suggested mitochondria. Their structural features were identical to the mitochondrial pattern seen with hematoxylin and eosin and the hemalum-phloxine-saffron stains. The observations of Nachlas and co-workers[27] also support the contention that mitochondria are the specific recipients of formazan deposition.

Cameron,[11] by ultracentrifuge and biochemical studies, demonstrated that succinic dehydrogenase enzymes in cells were limited to the mitochondria. It is generally accepted that tetrazolium in the presence of a succinate substrate is reduced only by succinic dehydrogenase;[29] thus it is reasonable to assume that the formazan deposition observed occurred only in mitochondria. The concentration of the deposit adjacent to the basement membrane of the convoluted tubule may have indicated the location of increased numbers of mitochondria, increased dehydrogenase content or both. The somewhat weaker staining in the terminal third of the proximal convoluted tubule reflected both a decrease in number and staining intensity of the granules. On the basis of these observations

it is tempting to assume that the terminal third of the proximal convoluted tubule has less oxidative enzymatic activity than the remainder. However, histochemical observations and physiologic activity, although related, are not necessarily entirely comparable.

The appearance of nuclei in tetrazolium-stained sections is of some interest. The mild staining of nuclear areas was obviously not due to the nuclei themselves. Because the thickness of the frozen sections was greater than that of the nuclei, cytoplasm lay above and below them. However, a nucleus was occasionally seen as a completely stain-free area. Glomeruli never exhibited any formazan deposition, indicating the passive role glomeruli play in filtration. The complete absence of activity in the renal pelvic epithelium was noted; Nachlas and co-workers,[27] however, reported weak staining in this location.

The Kidney in the Mercury Bichloride-treated Rat

Observations

In the rats examined 1 and 2 hours after the injection of mercury, no abnormal changes were noted in either the conventional or the tetrazolium-stained sections. However, at 3 hours in the inner cortical zone groups of 2 to 3 terminal segments of proximal convoluted tubules showed mild mitochondrial irregularity and early nuclear pyknosis. The tetrazolium-stained sections revealed no reduction in formazan deposition. At 4 hours there was no significant increase in the degenerative lesions as shown with conventional staining. However, tetrazolium-stained tissues exhibited a mild focal decrease in the intensity of formazan deposition in the inner cortical zone (Fig. 4).

At 5 hours conventional staining showed some increase in the number of degenerating terminal segments, mild smudging of mitochondrial detail and increasing prominence of nuclear pyknosis. The extent of alteration in tetrazolium-stained sections was no greater than at 4 hours. By 6 hours there was increased cytoplasmic eosinophilia in the terminal segments and some loss of nuclear detail; the mitochondria appeared a little larger than normal. The extent of alteration shown in the tetrazolium-stained sections was as great as that in conventional sections; some of the tubular epithelium showed a mild decrease in formazan deposition, and in some there was an increase in the size of stained organelles (Fig. 5). This was comparable to the increased size of mitochondria seen in conventional sections.

At 9 hours an increase in the size of foci and in the number of altered tubules was accompanied by occasional necrotic cells. The majority of cells had lost adhesion to each other and to the basement membrane. Although the tetrazolium stain also showed loosening of the epithelium,

formazan deposition, rather than being decreased in these cells, was actually increased (Fig. 6). At 12 hours naked eye examination of the sections revealed a mild and somewhat focal reduction in staining intensity in the inner cortical zone in sections stained with both techniques. About 75 per cent of the terminal segments of proximal convoluted tubules were necrotic; the remainder exhibited some mitochondrial irregularity. There was now a mild decrease in the intensity of formazan deposition in the inner cortical zone (Fig. 1B). Small scattered groups of the more proximal portions of proximal convoluted tubules in the outer cortical zone exhibited mild focal alterations.

Discussion

Histologically, the earliest lesions induced by mercury were noted at 3 hours, mitochondrial and nuclear changes commencing concurrently. Histochemically, the earliest alterations were observed at 4 hours and were characterized by a mild, focal reduction in formazan pigment deposition. Thus, in this study, decrease in dehydrogenase activity indicated by tetrazolium staining did not precede histologically demonstrable lesions. Wachstein and Meisel [30] and Rennels and Ruskin [31] had found reduced formazan deposition within 1 hour after the injection of a mercurial compound. However, these authors used larger amounts of mercury, a different compound (mercuhydrin), and a different tetrazolium for staining (neotetrazolium). Wachstein and Meisel's observation [20] that the extent of enzymatic alteration at a given time was greater than that of histologic change was not substantiated.

Mustakallio and Telkkä,[24] using mercury bichloride and staining with neotetrazolium, noted both histologic and enzymatic alterations at 4 hours. These findings were closer to ours and suggested that injury to dehydrogenase enzyme systems might not be the primary effect in mercury nephrotoxicity. With the tetrazolium method for succinic dehydrogenase activity, the deposition pattern of formazan granules in mercury nephrotoxicity is identical to that seen in mitochondria with conventional staining. Before their deposition is diminished, formazan particles become larger and more irregular than normal; this is also the case with mitochondria. Only after there is some loss of mitochondrial histologic detail and an accompanying homogeneous cytoplasmic eosinophilia is there a decrease in dehydrogenase activity. Complete loss of enzyme activity does not occur until the cell has been shed into the lumen and has undergone granular degeneration.

These findings controvert the assumption that mercury primarily damages succinic dehydrogenase enzymes to effect the eventual cell destruction. Wachstein and Meisel [19] supported this assumption by their

observation that the extent of enzyme alteration was greater than that indicated by histologic staining. However, this was, at best, a subjective observation and might have been the result of differences in shrinkage of sections prepared for routine and enzymatic stains. Handley and Lavik [16] suggested that mercurial diuresis was attributable to a depression of succinic dehydrogenase. However, Fawaz and Fawaz [32] injected mercurial diuretics into rats and found that at the height of diuresis, kidney slices showed no change in oxygen consumption or succinic dehydrogenase activity as determined by the Warburg flask. This would agree with our observation that a reduction in the intensity of formazan production occurred only after the onset of irreversible cellular damage. Also in agreement was the work of Cafruny, Farah and di Stefano,[33] who found that mercurials inhibited sulfhydryl groups of tissue proteins rather than those of enzymes.

While oxidation-reduction enzymes of mitochondria are not primarily affected by mercury, the mitochondria themselves are. Cameron [11] theorized that carbon tetrachloride attacks mitochondria of liver cells physically so that they lose their power of retaining enzyme co-factors. This leads to disorganization in the chain of enzymes which form the tricarboxylic acid (Krebs) cycle. This theory may also be applicable to mercury damage of mitochondria; we have observed mitochondrial abnormality before a reduction in succinic dehydrogenase activity became manifest.

Recent studies of serum enzyme levels in patients with myocardial infarction suggest that decrease in tetrazolium staining in damaged cells may not be due solely to destruction of enzyme systems. Jennings, Kaltenbach and Smetters [34] found significant increases in the levels of oxaloacetic transaminase, lactic dehydrogenase and succinic dehydrogenase in the serum of dogs with experimental myocardial ischemia. White [35] found a similar increase in serum lactic dehydrogenase in patients with myocardial infarction. Therefore, it appears likely that a decrease in the tetrazolium staining of mercury-damaged renal tubular cells is due, in part at least, to the release of enzyme from disrupted mitochondria. About 30 years ago Moore and co-workers [5] suggested that mercury bichloride produced tubular damage by its adsorption to the surface of mitochondria, thus "poisoning" the interface and later killing the cell. The present experimental results strongly suggest that the mitochondrial membrane is indeed the cytologic structure primarily damaged in mercury nephrotoxicity.

Summary

In order to determine the intracellular structure primarily damaged in mercury poisoning, mercury bichloride solutions were injected intra-

muscularly into the thigh muscles of rats. At hourly intervals, kidneys were examined by both conventional histologic methods and histochemical staining for succinic dehydrogenase activity utilizing tetrazolium-succinate incubating solutions. Although the earliest histologic and enzymatic alterations were noted in mitochondria, histologic lesions preceded enzymatic changes by at least one hour. Only after irreversible mitochondrial disintegration was there complete absence of succinic dehydrogenase activity. It is, therefore, suggested that mercury initiates cellular destruction by combining with the sulfhydryl groups of protein in the mitochondrial membrane. Disintegration of mitochondria then results in loss of enzyme activity. Presumably enzymes are released into the blood stream since this is manifest in cardiac and hepatic cellular destruction.

The observations may have some significance in understanding the action of mercurial diuretics. One widely accepted theory has it that these agents act by inhibition of succinic dehydrogenase. Our observations, however, indicate that even a toxic dose of mercury does not initially decrease enzymatic activity. This would suggest that mercury diuresis is not due to inhibition of oxidation-reduction enzymes.

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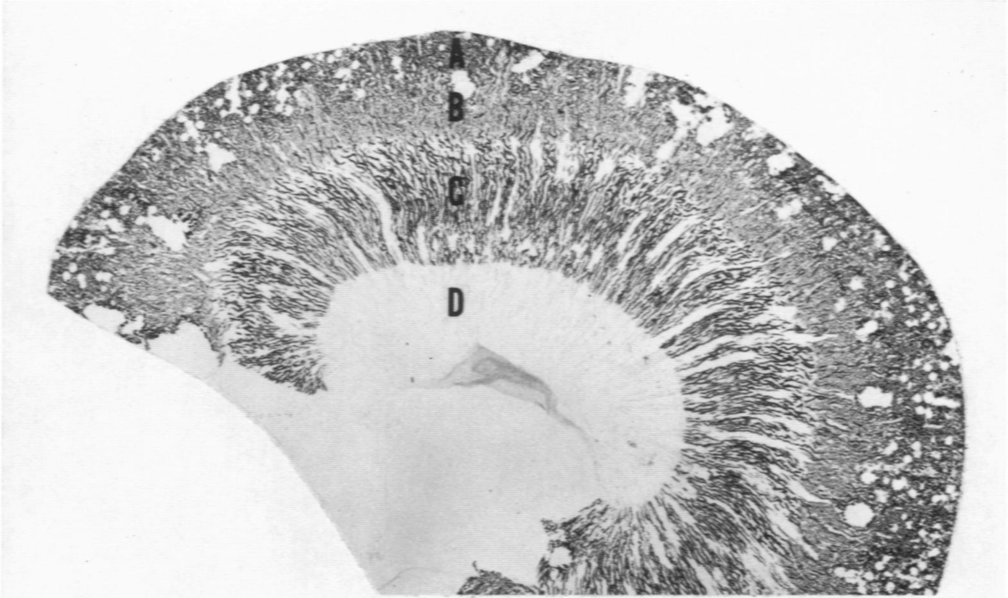
[*Illustrations follow*]

LEGENDS FOR FIGURES

All photomicrographs were prepared from sections stained with Nitro-BT.

FIG. 1A. Hemisection of a normal rat kidney stained with tetrazolium. There are 4 zones with decreasing intensity of staining from capsule to pelvis. A. Outer cortex. B. Inner cortex. C. Outer medulla. D. Inner medulla. Glomeruli appear in the outer cortex as empty "vesicles." × 8.

FIG. 1B. Hemisection of a rat kidney 12 hours after the intramuscular injection of mercury bichloride (0.005 mg. per gm.). There is mild and a somewhat focal decrease in formazan deposition in the inner cortical zone as compared to the normal. × 8.



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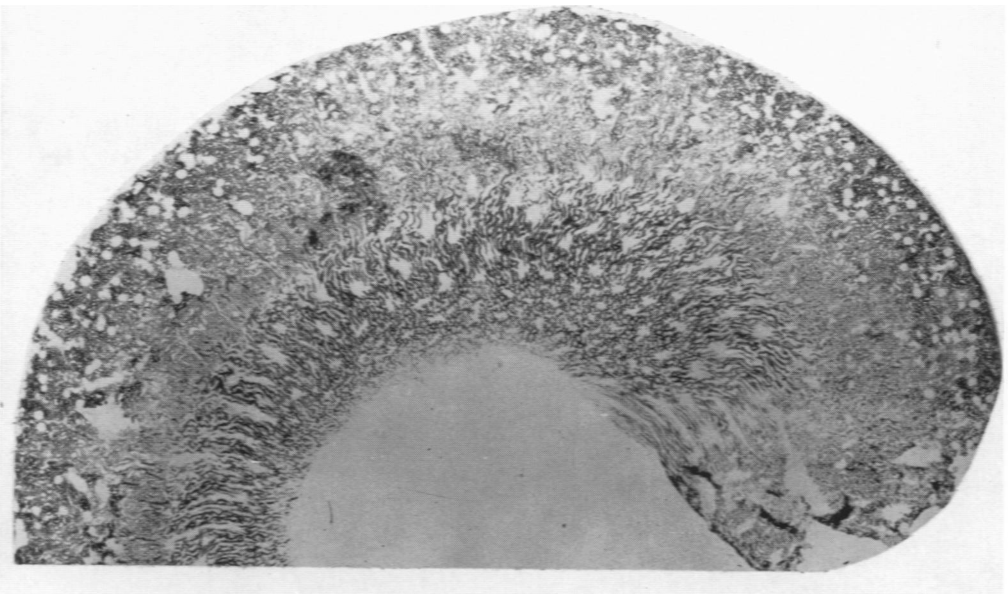

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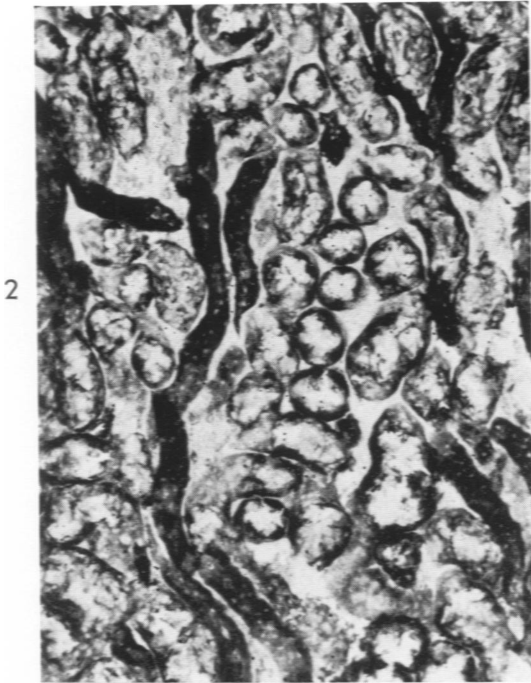
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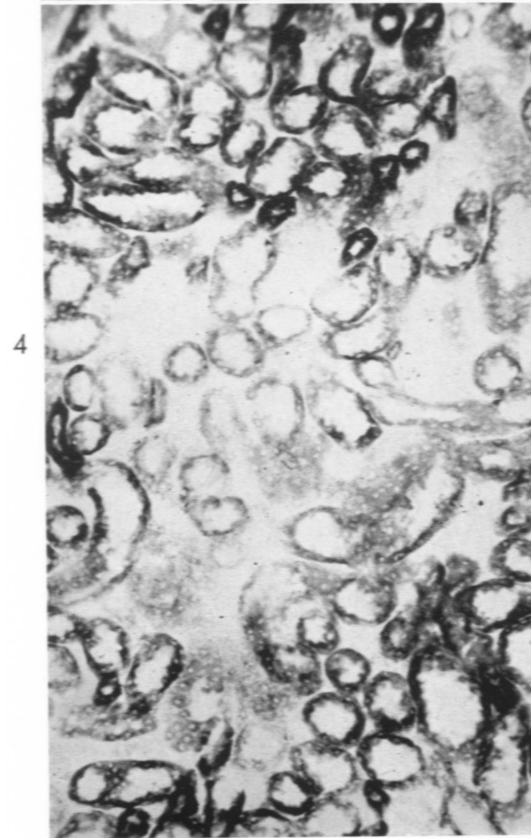
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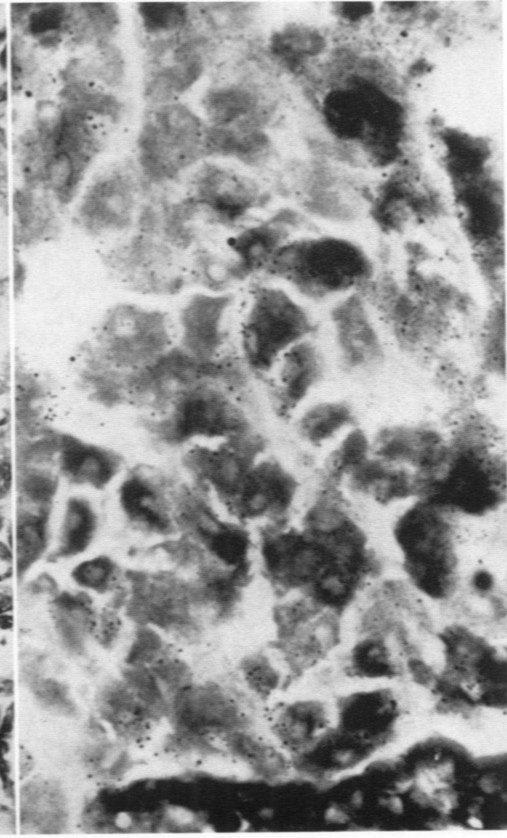
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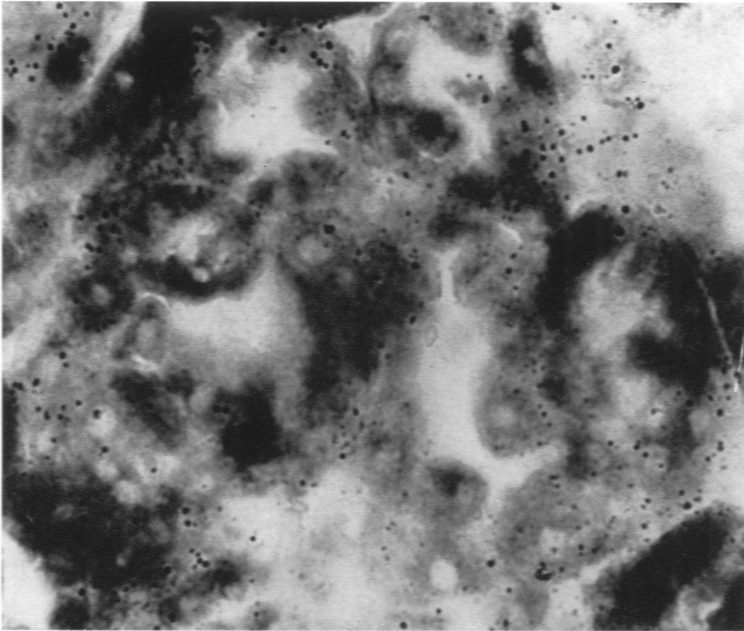

FIG. 2. Inner cortical zone of a normal rat kidney. Intensely staining loops of Henle contrast with the paler terminal segments of proximal convoluted tubules. × 100.

FIG. 3. Junction between the outer and inner medullary zones in a normal rat kidney. The intensely stained lower ends of the loops of Henle appear above. Below are continuations of collecting tubules which are lined by faintly staining cells. × 100.

FIG. 4. Inner cortical zone of a rat kidney 4 hours after the injection of mercury bichloride. A small focal area exhibits mild reduction in formazan deposition in the terminal segments of proximal convoluted tubules. × 100.

FIG. 5. Inner cortical zone of a rat kidney 7 hours after the injection of mercury bichloride. Although there are small zones of decreased formazan deposition, many of the cells in the terminal segments of proximal convoluted tubules contain enlarged organelles which appear as very fine dark punctate dots. × 400.

FIG. 6. Inner cortical zone of a rat kidney 9 hours after the injection of mercury bichloride. Many of the cells in the terminal segments of the proximal convoluted tubules have lost their attachment to the basement membrane. They nonetheless exhibit further increase in the size of formazan deposits. × 400.