# LYSOSOME INDUCTION IN EXPERIMENTAL POTASSIUM DEFICIENCY

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Experimental hypokalemia leads to alterations in renal function and structure in the laboratory mouse and rat.[1] Among these changes are an impaired mechanism for concentration of urine, an increased proteinuria, and vacuolar and granular changes in the cytoplasm of renal tubular epithelium. The striking and unique accumulation of hyaline granules or droplets in the renal papillae of potassium-depleted mice and rats is a characteristic finding in this experimental situation. These granules, first noted in mice by Liebow, McFarland and Tennant in 1941,[2] have been studied in recent years by a variety of techniques, including histochemistry[3-6] and electron microscopy.[7-9] Differences in interpretation of histochemical and electron microscopic data have led to two divergent views of the identity of the characteristic granules; one, that they are altered mitochondria,[5,7,9] and two, that they are the result of an alteration in unspecified but non-mitochondrial cytoplasmic constituents.[4,8]

Our purpose in this study has been to analyze further the nature and origin of these distinctive granules in experimental potassium deficiency. Results of our investigations using electron microscopy, light microscopy, histochemistry, and enzyme chemistry led us to identify these particles as members of a class of cytoplasmic particle isolated by de Duve, Pressman, Gianetto, Wattiaux and Appelmans[10] and named by them "lysosomes." We have reported our findings in a preliminary way elsewhere.[11,12] In this report we present a comprehensive account of our observations which, through morphologic, histochemical, and chemical data, establish the identity of these granules in experimental potassium depletion as lysosomes.

## MATERIAL AND METHODS

Male Wistar rats weighing 175 to 200 gm. at the beginning of the experiment were fed a low potassium diet with added vitamins (Nutritional Biochemicals Corporation, Cleveland, Ohio). Composition of diet as obtained from the manufacturer: Corn starch, 64 per cent; casein, 30 per cent; butterfat, 3.5 per cent; calcium carbonate, 1.3 per cent; sodium chloride, 1 per cent. This formula was supplemented with the

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following amounts of vitamins added to each 100 pounds of diet: Vitamin A concentrate (200,000 units per gm.), 4.5 gm.; vitamin D concentrate (400,000 units per gm.), 0.25 gm.; alpha tocopherol, 5 gm.; ascorbic acid, 45 gm.; inositol, 5 gm.; choline chloride, 75 gm.; menadione, 2.25 gm.; p-aminobenzoic acid, 5 gm.; niacin, 4.5 gm.; riboflavin, 1 gm.; pyridoxine hydrochloride, 1 gm.; thiamine hydrochloride, 1 gm.; calcium pantothenate, 3 gm.; biotin, 20 mg.; folic acid, 90 mg.; and vitamin $B_{12}$, 1.35 mg. The diet on analysis contained 8 mEq. potassium per kg., 340 mEq. sodium per kg., and 7 mEq. magnesium per kg. To the diet were added 100 mg. each of magnesium carbonate and magnesium chloride per 100 gm. of diet. The control animals were pair-fed the same diet, including the magnesium supplement, to which had been added 0.5 gm. each of $K_2HPO_4$ (Sorenson's salt) and KCl per 100 gm. of diet.

A total of 20 experimental and 9 control animals were killed 1, 2, 3, 4 and 8 days and 4 weeks after beginning the experiment. In addition, 3 experimental rats which had been fed the potassium-deficient diet for 4 weeks were replenished with potassium by feeding the control diet and were killed 1, 2, and 3 weeks after potassium had been restored to their diet.

The rats were anesthetized with ether, bled, the kidneys quickly dissected, papillae removed, and representative portions of the cortex and outer medulla taken. The tissues taken for electron microscopy were fixed in cold 1 per cent osmium tetroxide buffered with veronal acetate to pH 7.25 to 7.4. Tissues prepared for electron microscopic demonstration of acid phosphatase were handled by the method described by Miller. [13] They were fixed in glutaraldehyde buffered with sodium cacodylate to a final pH of 6.8, and sucrose was added to a final concentration of 7.5 per cent; the fixative in this form was suitable for sections of renal cortex, but when used for the papillae it was found to cause excessive tissue damage. It was found that the damage could be prevented by the addition of more sucrose to make a final concentration of 12 per cent.

Sections of papillae were cut at 40 $\mu$ on a Sartorius freezing microtome. Omission of the acetic acid wash in the Gomori acid phosphatase procedure produced no apparent change in degree or distribution of staining, and its elimination reduced the amount of tissue handling required. This is in accord with Desmet's finding that a 2-minute soaking in 2 per cent acetic acid was sufficient to dissolve out deposited lead and cause a false negative reaction.[14] The Gomori acid phosphatase technique was performed at pH 5; 2 controls were run: 1 consisted of the substrate mixture from which beta-glycerophosphate was omitted, and the other, the substrate mixture to which 0.01 M NaF, an inhibitor of acid phosphatase, was added.

Tissue blocks of papillae and the 40-$\mu$ sections used for the Gomori acid phosphatase reaction were embedded in Epon by the method of Luft.[15] Sections were cut on an LKB microtome with glass knives. Silver-gold sections were mounted on carbon-coated grids and examined with an RCA 2A electron microscope. All sections, including those stained by the Gomori method for acid phosphatase, were stained with lead by the method of Karnovsky [16] after preliminary trials with the lead stain showed that it did not influence the results of the acid phosphatase reaction.

For the chemical determination of renal papillary acid phosphatase, additional groups of rats were fed the experimental and control diets used above. Tissue acid phosphatase was determined by the method of de Duve and co-workers.[10,17]

## Results

Rats made potassium-deficient developed polyuria between the fifth and tenth days after the start of the experiment. Urine volumes were 3 to 4 times those of control rats. Proteinuria of 2 to 3 times the normal level developed on about the tenth day. Grossly, enlargement of the renal

papillae of the potassium-depleted animals was noticeable on about the third or fourth day, the enlargement becoming very prominent by the eighth day.

Both by light and electron microscopy there were numerous granules present by the third day after beginning the experiment. It was our impression that an increase in numbers of granules visible by electron microscopy occurred as early as 24 to 48 hours after the onset of the experiment in potassium-depleted as compared to control rats. No significant changes in glomeruli could be detected at any stage of the experiment. We found, as Spargo, Straus, and Fitch [8] have observed, that granules were present in cells of the papillary collecting tubules, in capillary endothelium and in interstitial cells of the papillae.

The electron microscopic appearance of cells in the papillary collecting tubules, ducts of Bellini, capillaries, and interstitium has been described for normal mice by Rhodin.[18] Similar appearances were found in the normal rat by Spargo and co-workers.[8] For the sake of this study, some features relating to cell surface and cytoplasmic contents noted in the control rats deserve emphasis.

The normal columnar cells of the collecting tubules showed a microvillar appearance along the lumen border (Fig. 1). Higher magnification of the cytomembranes at this border showed a unit membrane structure and microvilli (Fig. 2). Usually, an irregular extraneous coating was present on the tubular surface, within which were numerous dense granules measuring 50 to 100 Å (Fig. 3). These granules appeared in both control and potassium-deficient rats, but seemed more noticeable in the latter.

Terminal bars were evident at the lateral borders of adjacent cells, and interdigitation of lateral cell membranes was noted. The cytoplasm contained vesicles (Fig. 2), and relatively small numbers of small mitochondria containing few cristae were present. The endoplasmic reticulum was sparse, with only a few rough and smooth-profiled vesicles seen. The Golgi apparatus was frequently prominent (Fig. 1).

Rarely, dense bodies, usually smaller than the mitochondria and exhibiting a single outer membrane rather than the mitochondrial double membrane, could be seen. The basal infoldings of tubular epithelium were shallow compared to those in more proximal portions of the nephron. The tubular basement membrane was a thin condensation which separated tubules from either peritubular capillaries or from the interstitium. In the interstitium were scattered cells containing in their cytoplasm a few mitochondria and varying numbers of irregular, homogeneous, dense granules of a configuration easily distinguishable from the granules or

droplets of the potassium-deficient rat. These granules were composed of lipid or lipoprotein; their function is unknown.

Major morphologic changes occurred in the cytoplasm of all renal papillary cells with the formation of the characteristic granules. The numerous granules in rats kept on potassium-deficient diets for 1 month occupied a large part of the cytoplasmic space and seemed to increase its volume. They varied in size, reaching almost 5 $\mu$, and displayed a variety of forms (Fig. 4). Mitochondria were easily distinguished from granules although the former sometimes exhibited minor degrees of change, generally in the form of internal rearrangement of cristae. Similar granules or droplets occupied large parts of the cytoplasm of capillary endothelium and interstitial cells (Fig. 5). In addition, an occasional intracytoplasmic myelin figure was seen (Fig. 5). Potassium deficiency caused no significant separation of lateral cell borders of the tubular epithelium during the period of our experiments.

From examination of papillae in potassium-depleted rats sequentially killed, there appeared to be a general pattern discernible in lysosome formation. The characteristic lysosomal granules displayed an outer cytomembrane of unit type (Figs. 6 to 8) which was often not complete around the granule's circumference. The material composing the granules might take the form of dense granules 30 to 100 Å in diameter with vesicular bodies measuring 200 to 500 Å. Occasional clusters of dense granular material measuring 50 to 250 Å were seen within the granules and in the cytoplasm; these resembled ribonucleoprotein (RNP) granules. In some granules, particles of unit membrane were occasionally included in the substance (Fig. 7). There was usually a rough filamentous quality to the external surface of the granules. When compared to adjacent mitochondria, the differences were easily apparent (Figs. 4 and 8). There were also structures composed almost entirely of aggregates of vesicles with little or no detectable limiting membrane; such multivesicular bodies had vesicles like those in the membrane-limited granules (Fig. 8).

In animals fed a low potassium diet for 4 weeks and returned to a stock diet, the granules began to decrease in size and number so that some animals were almost free of them at the end of 1 week. In some of the rats, however, dense bodies and granules were present in numbers greater than in control rats as late as 3 weeks after return to control diet. The dense bodies could be nearly 1 $\mu$ in diameter with a unit type of limiting membrane and a dense interior separated from the membrane by a clear rim and frequently containing focal dense or vacuolated areas (Fig. 9). It appeared from examination of the resolving granules that they underwent progressive decrease in size with increasing homogenization of the contents.

Examination of sections with the Gomori acid phosphatase reaction revealed small numbers of granules containing dense crystalline deposits of lead (Fig. 10). The Gomori reaction applied to sections of control papillae revealed rare dense bodies with membrane-localized reaction product (Fig. 11). No dense deposits were ever observed on the mitochondria. No deposition of crystalline lead was seen in control enzyme preparations from which the substrate was omitted or to which fluoride inhibitor was added. A greater proportion of granules exhibiting the deposition of the lead reaction product was encountered in interstitial cells and in capillary endothelium than in papillary tubular cells (Fig. 12) even though the granules in interstitial cells and in capillary endothelium were morphologically identical to the tubular epithelial granules.

Chemical determination of the tissue acid phosphatase level disclosed significant differences between the renal papillae of control and experimental animals (Table I). It was not possible to estimate how much of

TABLE I

PHOSPHATASE ACTIVITY IN RENAL PAPILLAE OF RATS *

| | Total acid phosphatase | Free acid phosphatase | Bound acid phosphatase |
|---|---|---|---|
| Controls | 86 ± 7 † | 78 ± 3.4 | 12 ± 6.6 |
| | n = 8 ‡ | n = 6 | n = 6 |
| Potassium-deficient | 557 ± 52 | 263 ± 52 | 242 ± 21 |
| | n = 13 | n = 8 | n = 8 |

* The activities are expressed as μg. of inorganic phosphorus liberated from beta-glycerophosphate by 100 mg. wet weight of renal papillary tissue per hour.

† Mean ± standard error of mean.

‡ n = number of papillae analyzed.

this enzyme activity resided in granules of the tubular cells and how much was present in those of interstitial and endothelial cells.

As a further check on the lysosomal nature of the granules, beta-glucuronidase, another enzyme considered to be associated exclusively with lysosomes, was estimated chemically in renal papillae from control and experimental rats. Preliminary work carried out in collaboration with Dr. John Ashton in this department has indicated that, as in the case of the acid phosphatase, significant increases of beta-glucuronidase over control values were found in the experimental animals.

## DISCUSSION

The granules or droplets forming in the cells of renal papillae during potassium depletion were first noted in the mouse by Liebow and coworkers in 1941.[2] They apparently considered them to be the result of an alteration in the state of the cytoplasm since they referred to their

presence as "colloid change." Spargo in 1954[3] called attention to the appearance of the granules in the papillary collecting tubules in the potassium-depleted rat and attempted to demonstrate their nature, using histochemical methods. He concluded that they were a glycoprotein which he called hyaline droplets. Further investigation of their nature was undertaken by histochemical methods. Although observations were often in fair agreement, the interpretation of the results was difficult. Craig and Schwartz[4] considered the droplets to be a lipoprotein complex arising within the cell, while Pearse and MacPherson[5] concluded that they were derived from mitochondria by the addition of a polysaccharide-containing protein.

Early electron microscopic studies of the renal papillae in the potassium-deficient rat were thought to show the transformation of mitochondria into the characteristic granules.[7] Spargo and co-workers,[8] on the other hand, denied their origin from mitochondria. They suggested that they were not necessarily related to any particular cytoplasmic structure but were formed as a result of a change in the protoplasmic state possibly associated with the osmolality of the renal papillary interstitium. The droplets were shown to react with anti-whole-rat-serum rabbit globulin. The same investigators also suggested that urinary mucoproteins might represent a secondary addition to the droplets.

Using T-1824 injections in potassium-depleted rats, Morrison and Gardner[19] demonstrated coloring of the granules in the renal papillae while there was a diminution of staining in the proximal convoluted tubules of experimental rats as compared to controls. The T-1824 appeared in the urine of potassium-deficient rats but not in the controls, and there was an increase in the amount of protein excreted in the urine of the former compared to controls. These investigators interpreted these findings to mean that serum protein, perhaps escaping through the glomerulus, entered the granules. Additional histochemical studies carried out by Gasic and Morrison[20] showed that the PAS positivity of the granules was due to a polysaccharide other than glycogen and that acid mucopolysaccharides stained by the Hale iron method were included in them. Additional histochemical tests were interpreted by these authors as suggesting that the acid mucopolysaccharide in all likelihood contained sialic acid.

Most investigators have found increased amounts of acid phosphatase in the renal papillae in potassium-deficient rats by both the Gomori method and the azo dye technique.[4-6] Acid phosphatase is among the hydrolytic enzymes contained in cytoplasmic organelles recovered from fractionated liver cells.[10] These organelles, which de Duve called "lyso-

somes," differ morphologically and biochemically from other cellular particles.[21]

Acid phosphatase in the rat renal cortex has been shown to reside in bodies referred to as "phagosomes" by Straus.[22,23] These have properties similar to those of the hepatic lysosomes of de Duve. The demonstration of the lead reaction product of the Gomori acid phosphatase reaction in characteristic bodies in proximal convoluted tubular epithelium by electron microscopy[13] lends support to Straus's idea that these bodies in proximal tubules are lysosomes or phagosomes since acid phosphatase is generally accepted as providing a marker for the lysosomes.[24,25]

By electron microscopy we have demonstrated that the granules formed in the renal papillae of potassium-deficient rats were morphologically similar to lysosomes in other situations. Then using electron microscopic histochemical methods, we have shown the localization of the lead reaction product in the Gomori method to the specific granules. This substantiated the impression obtained from light microscopy with both the Gomori method and the Burstone azo dye technique.[26] In parallel chemical determinations of tissue enzyme content in renal papillae, we demonstrated a striking increase in the amount of bound acid phosphatase in potassium-depleted rats as compared to controls (Table I).

It is concluded that the morphologic observations, protein content, T-1824 absorption, mucopolysaccharide and enzyme content permit the positive identification of these granules as lysosomes. None of the data suggests a mitochondrial origin. Since these cytoplasmic bodies are either absent or present only in very small numbers in the cells of the normal rat renal papillae, we have thus identified an experimental procedure which results in the new formation of large numbers of lysosomes.

## Summary

Rats made potassium-deficient by feeding a low potassium diet developed characteristic granules in the cytoplasm of cells in the renal papillae. These granules could be seen in large numbers within a few days of initiating the experimental diet and disappeared within 1 to 3 weeks after correcting the potassium deficiency. Examination of renal papillae by electron microscopy showed the granules to differ from mitochondria and to appear similar to lysosomes. Light microscopy and electron microscopic histochemistry demonstrated localization of acid phosphatase in them. Chemical analysis of the renal papillae in control and experimental animals revealed a marked increase in acid phosphatase in potassium-deficient rats. Lysosomes were thus identified as the characteristic granules in the renal papillae in potassium-depleted rats. The study has also demonstrated a method for the experimental production of lysosomes.

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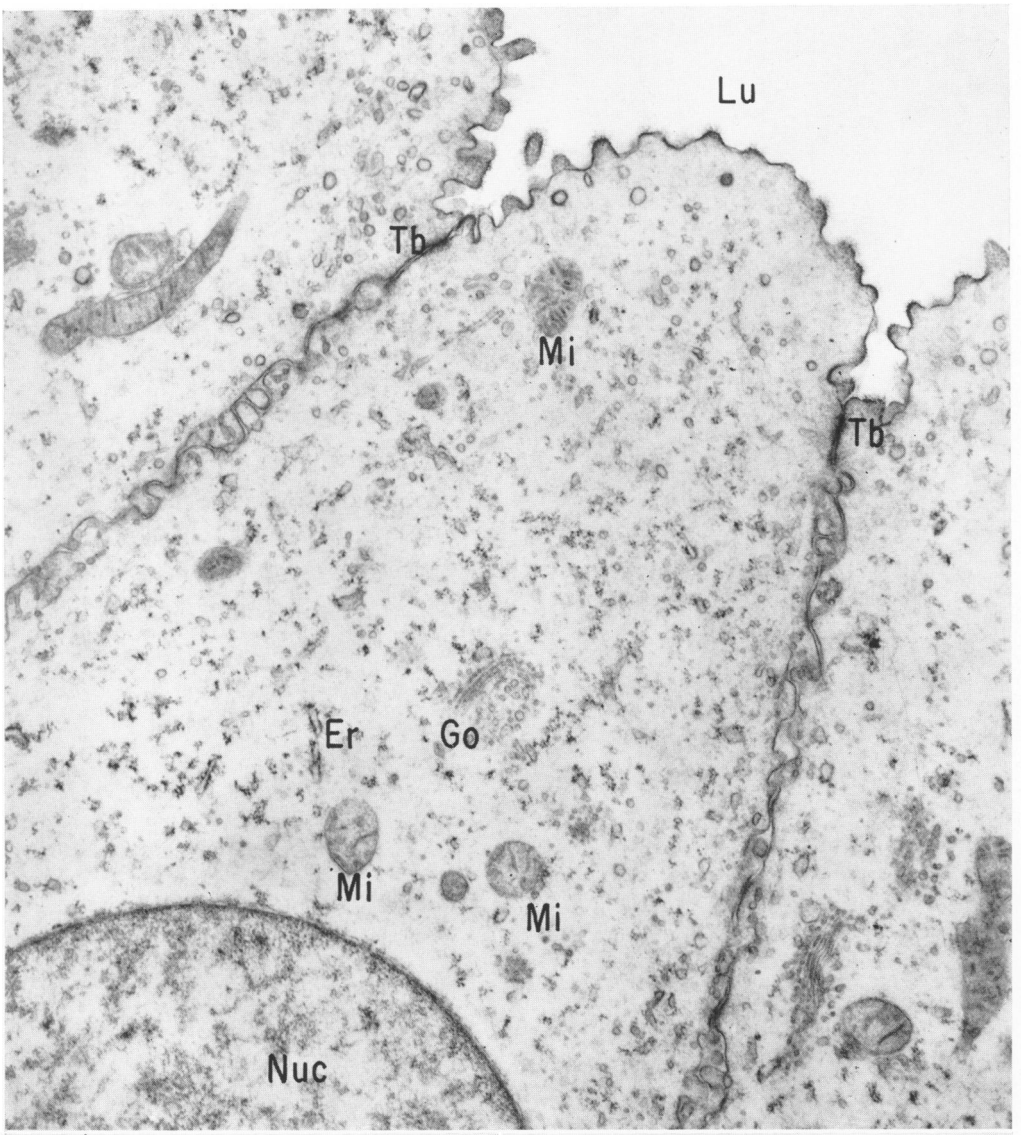
[ *Illustrations follow* ]

## Legends for Figures

FIG. 1. Control rat. Apical portions of 3 normal papillary collecting tubule cells with the microvillar cell borders extending into the tubular lumen (Lu). The interdigitating lateral borders of the cells show dense areas at the terminal bars (Tb). Within the cytoplasm may be seen mitochondria (Mi), endoplasmic reticulum (Er), Golgi apparatus (Go), and numerous vesicles. Part of the nucleus (Nuc) is at the bottom. × 12,000.

FIG. 2. Control rat. A portion of the apical cytoplasm in a normal papillary collecting tubular cell. Extending into the lumen (Lu) are fine microvillar processes of plasma membrane with a unit membrane structure. Vesicles (ves) of varying density and granules of different sizes are seen in the cytoplasm. × 55,000.

FIG. 3. Rat on a potassium-deficient diet for 4 days. Apical portion of a papillary collecting tubular cell. An irregularly dense extraneous coating (EC) is seen on the lumen side of the plasma membrane in microvilli. Within this coating are dense granules measuring 50 to 100 Å in diameter; these also appear along the plasma membrane within the cytoplasm and the vesicles. × 55,000.

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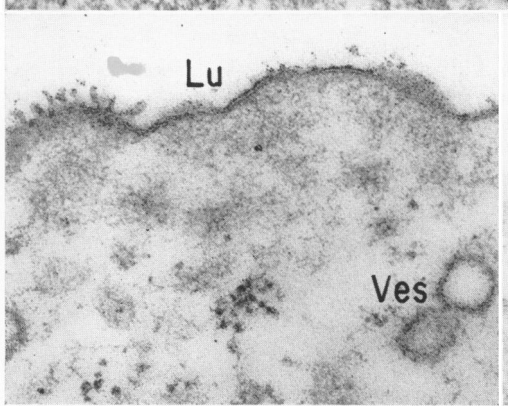


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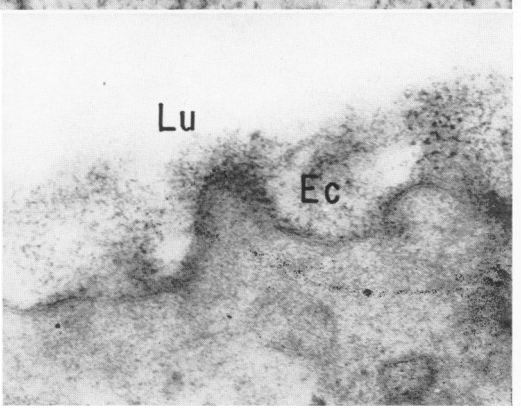


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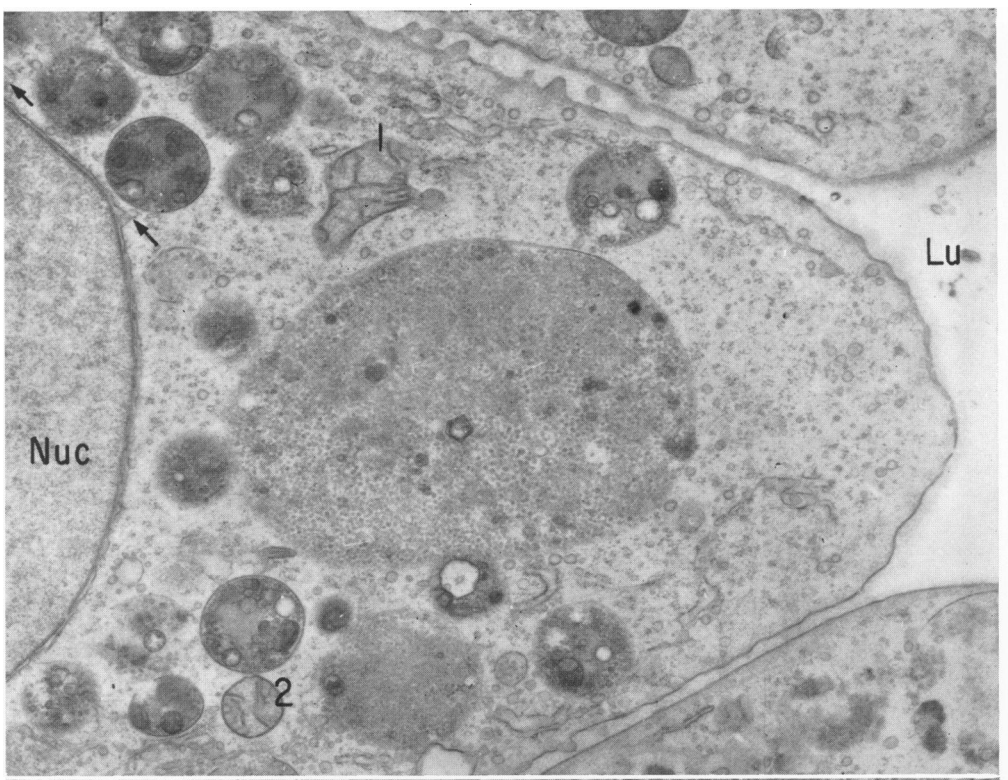
FIG. 4. Rat on a potassium-deficient diet for 1 month. The cytoplasm in the apical portion of a papillary collecting tubular cell appears to bulge so that microvilli appear flattened as they extend into the lumen (Lu). Lysosomes in the cytoplasm can be distinguished from mitochondria (1, 2). There are outpouchings (arrows) of the nuclear envelope (Nuc). × 12,000.

FIG. 5. Rat on a potassium-deficient diet 1 month. A portion of capillary (Cap) in the papilla. Within the cytoplasm of an endothelial cell (End) are a mitochondrion (Mi) and numerous lysosomes. A single myelin figure is apparent (My). Adjoining the capillary basement membrane (BM) are interstitial cells (IC) which contain irregular lipid droplets (arrows) in their cytoplasm (Cyt). Within the loose interstitial space many short fibrils may be seen. × 7,000.

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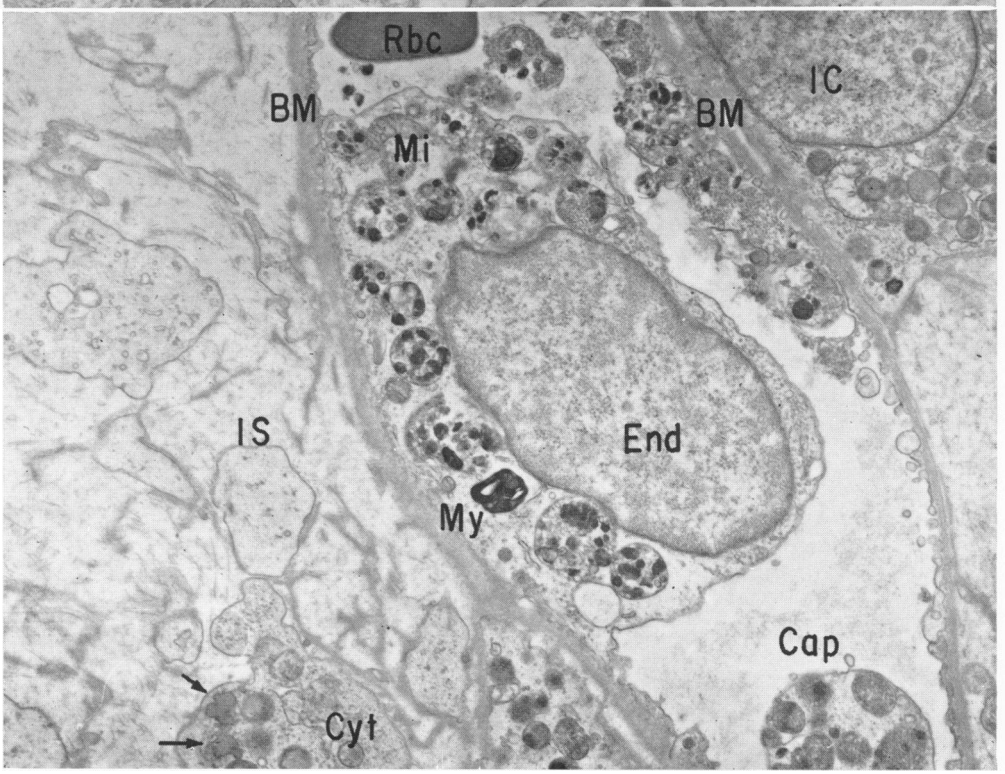


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FIG. 6. Rat on a potassium-deficient diet for 4 days. Lysosomes in a papillary collecting tubular cell. One lysosome (Ly 1) composed of clusters of granules measuring 30 to 100 Å is partially bounded by a membrane (arrows). × 52,000.

FIG. 7. Rat on a potassium-deficient diet for 8 days. Lysosome (Ly) in a papillary tubular cell. This is composed predominantly of vesicular bodies partially surrounded by a membrane and containing bits of membrane (arrow) within the body. Dense particles within the cytoplasm lie near the lysosome. × 46,000.

FIG. 8. Rat on a potassium-deficient diet for 4 weeks. Portion of papillary tubular epithelial cytoplasm. One lysosome (Ly 1) is composed of multiple vesicles and exhibits an almost complete surrounding membrane. Mitochondria (Mi) retain their characteristic structure and are easily distinguished from the lysosomes (Ly 1, Ly 2). Clusters of vesicles (MV) without limiting membranes, other vesicles, and dense granules are also seen. × 15,000.

FIG. 9. Rat on a potassium-deficient diet for 4 weeks and then fed the control diet for 1 week before sacrifice. Four lysosomes (1, 2, 3, 4) appear near the nucleus (Nuc) of a collecting tubular cell in the renal papillae. The lysosomal content is extremely dense with only a few dense particles and vacuoles (1 and 3). A thin rim separating the content from the limiting membrane is most clearly seen in 1. × 16,000.

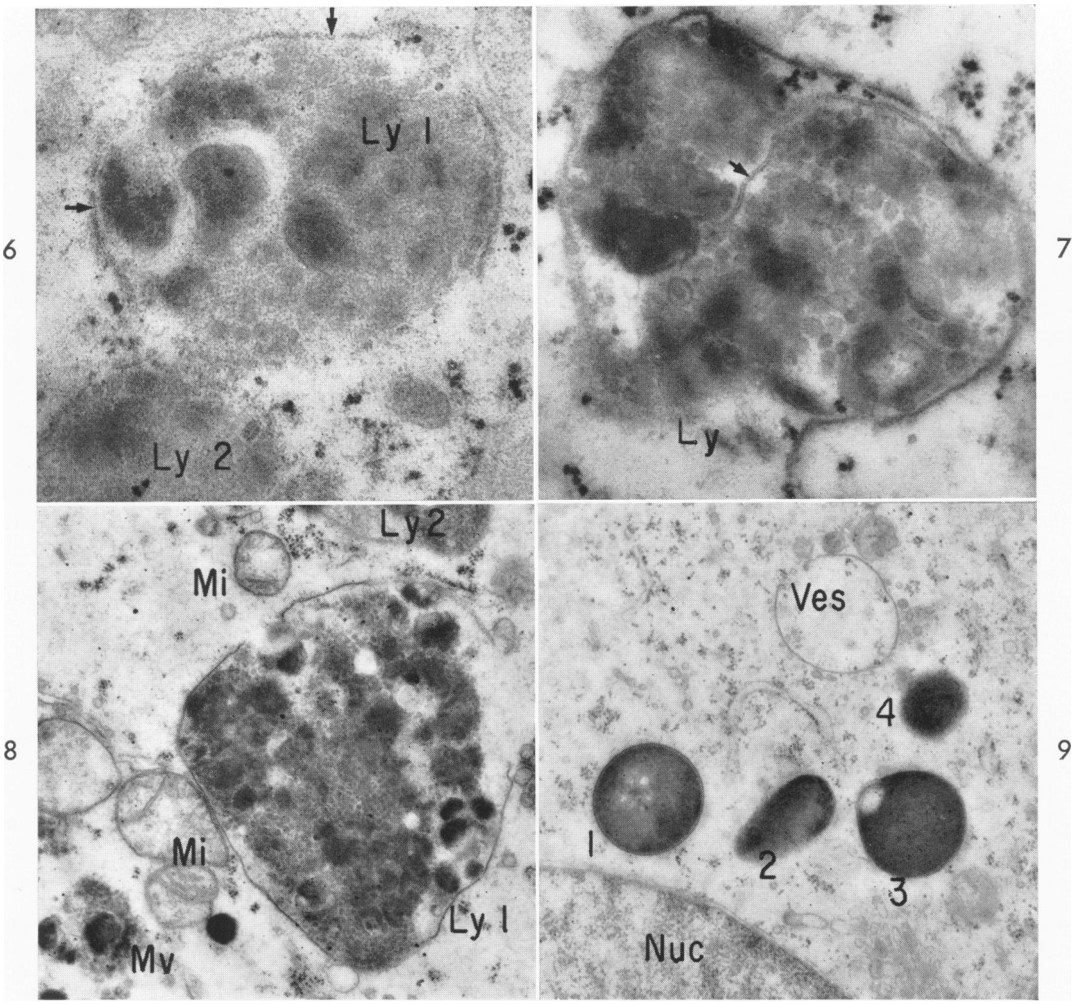


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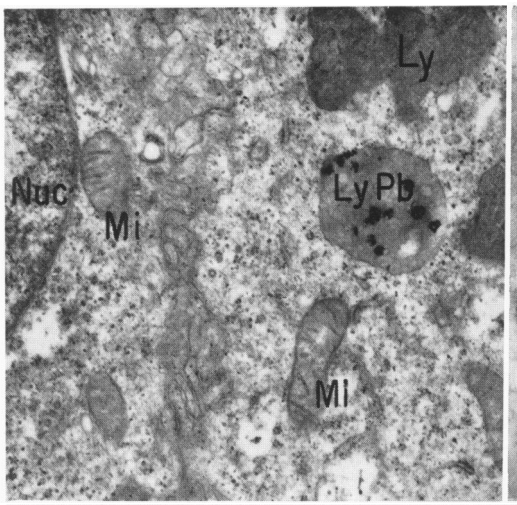
FIG. 10. Rat fed a potassium-deficient diet for 3 weeks. Parts of two adjacent cells from a papillary collecting tubule. No separation of the interdigitating lateral cell borders is seen. One lysosome (Ly Pb) exhibits lead deposition, reflecting the Gomori acid phosphatase reaction. The nucleus (Nuc), mitochondria (Mi) and other cytoplasmic structures are free of lead precipitate. Another lysosome (Ly) contains no reaction product. × 16,000.

FIG. 11. Control rat. Lysosomes (1, 2, 3, 4) in the cytoplasm of a normal collecting tubule cell. The Gomori technique for demonstrating acid phosphatase has resulted in lead deposition on a single lysosome (2). The other 3 lysosomes, the nucleus (Nuc), a mitochondrion (Mi), the cytoplasm and the cell lateral membranes are free of lead reaction product. × 22,000.

FIG. 12. Rat on a potassium-deficient diet for 3 weeks. Nuclei of interstitial cells (IC) cluster between basement membranes (BM) of collecting tubules in the renal papilla. Many of the lysosomes exhibit a lead reaction product. × 28,000.

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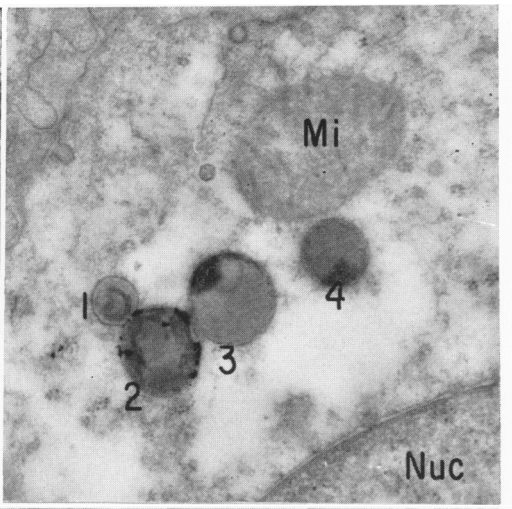


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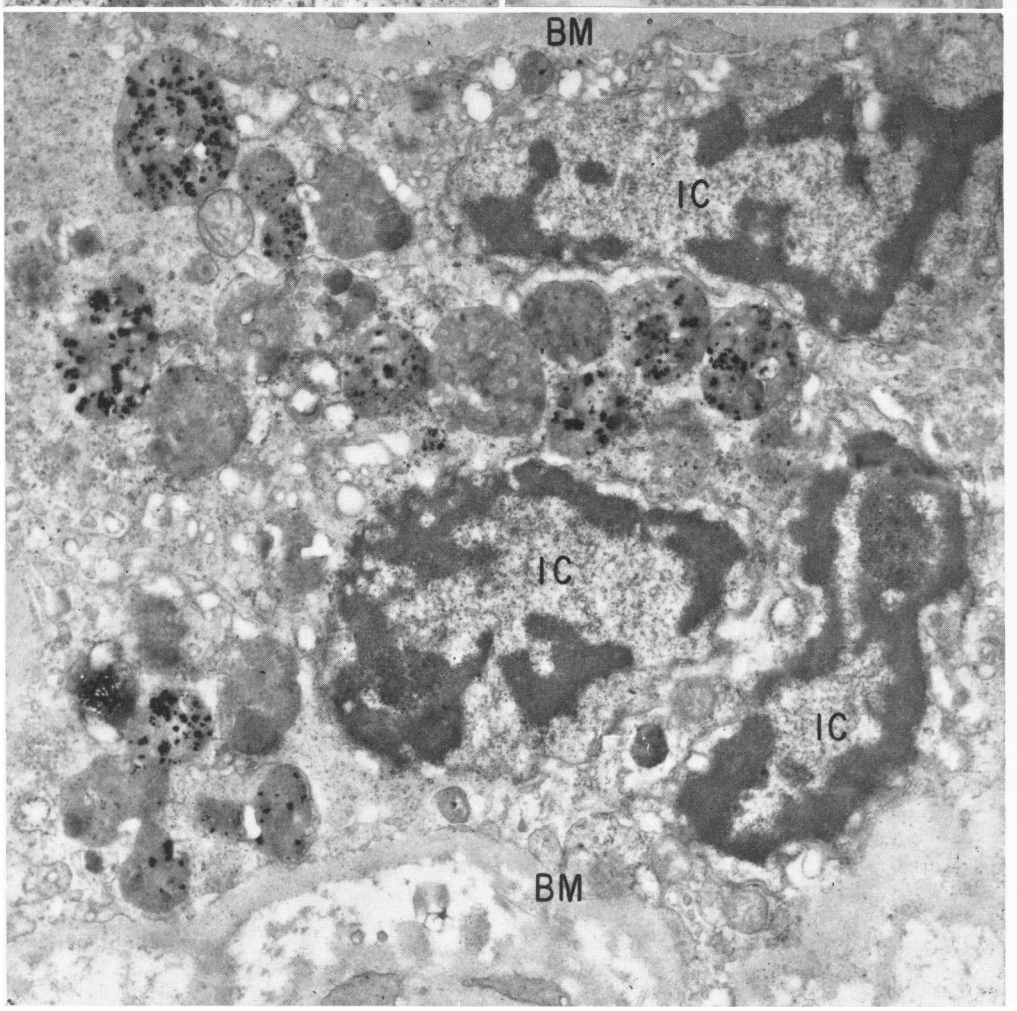


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