A COMPARATIVE CYTOCHEMICAL AND CYTOLOGIC STUDY OF VITAMIN D INDUCED NEPHROCALCINOSIS

DANTE G. SCARPELLI, M.D.; GILLES TREMBLAY, M.D.,* AND A. G. EVERSON PEARSE, M.D.

From the Departments of Pathology, The Ohio State University, Columbus, Ohio, and the Postgraduate Medical School of London, England

The renal lesions produced by hypervitaminosis D were first described simultaneously by Kreitmair and Moll[1] and Pfannenstiel[2] in 1928. Since then these lesions have been studied both experimentally and clinically by numerous investigators.[3-9] However, little is known about their pathogenesis. In fact, such basic questions as whether cell damage precedes the deposition of calcium or is caused by it remain unanswered at present. The results of this investigation show that the intracellular deposition of calcium is preceded by cell damage, as evidenced by cytochemical and cytologic alterations.

MATERIAL AND METHODS

Fifty-four male albino rats, weighing between 160 and 190 gm., were divided into 3 groups of 18 animals each. In each group 9 animals were given 50,000 units each of vitamin D_2 (Radiostol, British Drug Houses, Ltd.) daily by stomach tube. The remaining rats served as controls. An experimental and a control animal were sacrificed each day for 9 days. Groups 1 and 2 were used for cytochemical and cytologic investigations. The animals of group 3 were used in a second experiment to determine the morphologic stability of kidney mitochondria and the concentrations of calcium and citric acid in this tissue.

Cytochemical and Tinctorial Techniques

The kidneys were removed as rapidly as possible after death, mounted on metal chucks, and frozen within 20 seconds by dipping the base of the chuck in an acetone-solid CO_2 mixture. Fresh frozen sections of kidney were cut at 4 and 8 μ in a cryostat and mounted on cover slips. To demonstrate sites of diphosphopyridine nucleotide (DPN) diaphorase, triphosphopyridine nucleotide (TPN) diaphorase, isocitric dehydrogenase, alkaline phosphatase, and A type and B type indoxyl esterase

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activities, the following methods were employed: DPN diaphorase and TPN diaphorase by chelation of cobalt by the formazan of 3,5-diphenyl-2-(4,5 dimethyl-thiazol-2-yl) tetrazolium bromide with reduced DPN and TPN as substrates,[10] isocitric dehydrogenase by using Nitro-BT [2,2′-di-p-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride] [11] with DL isocitric acid lactone as substrate and oxidized DPN as coenzyme [12] (these reactions were carried out in media containing 7.5 per cent polyvinylpyrrolidone to preserve mitochondrial structure [13]); alkaline phosphatase by the azo dye method described by Grogg and Pearse [14] with the diazotate of 4-benzoyl amino-2:5-dimethyoxyaniline as the coupling salt, and indoxyl esterase by using o-acetyl-5-bromoindoxyl as substrate, according to the method of Holt and Withers.[15] Diethyl-p-nitrophenyl phosphate (E-600) was used to inhibit carboxylic acid B type esterases.[16] Incubation at pH 5.0 in the presence of E-600 showed sites of A type cathepsinlike esterase activity.[17] To demonstrate the presence of neutral fat, duplicate sections were stained with oil red O. In addition, serial sections were fixed in Helly's fluid and stained with Heidenhain's iron hematoxylin for the demonstration of mitochondria. Duplicate paraffin sections were stained with hematoxylin and eosin in the conventional manner, the Hotchkiss periodic acid-Schiff (PAS) reaction for 1,2 glycol groups, using diastase and saliva controls, and the Millon and Sakaguchi reactions for tyrosine and arginine-containing proteins.[18]

Biochemical Techniques

Alterations of mitochondrial structure were determined by the light-scattering technique of Cleland [19] and Raaflaub.[20] The kidney was stripped of its capsule, placed on ice and the corticomedullary area dissected free under a dissecting microscope. The tissue from these areas was diced and forced through a precooled perforated plate with 1 mm. holes. This was homogenized in a Potter-Elvehjem homogenizer in 0.44 M sucrose containing 0.01 M disodium ethylenediamine tetra-acetate (EDTA).[21] The resulting brei was centrifuged at 900 × g. The supernatant was then centrifuged at 7,000 × g, and the resulting mitochondrial pellet suspended in 0.3 M sucrose buffered at pH 7.3 with tris (hydroxymethyl) amino-methane (tris). Changes in optical density at 520 mμ were determined in a Beckman DU spectrophotometer at 10-minute intervals at 20° C. The serum and kidney tissue concentration of calcium was determined with a Beckman flame photometer according to the method described by Ballentine and Burford.[22] The citric acid concentration was determined colorimetrically by the method of Natelson, Pincus and Lugovoy.[23]

RESULTS

Results Obtained by Cytochemical and Tinctorial Methods

DPN Diaphorase, TPN Diaphorase, and Isocitric Dehydrogenase. Sites of DPN diaphorase, TPN diaphorase and isocitric dehydrogenase activity in the epithelium of the proximal convoluted tubules of control rats were localized as fine cytoplasmic deposits of blue-black formazan (Fig. 1). Under oil immersion these were resolved as a series of discrete 0.2 to 0.35 μ cobalt-formazan deposits distributed in rod-shaped mitochondria. Forty-eight hours after the administration of vitamin D, there was a decrease in the number of rod-shaped mitochondria (Fig. 2). In addition, the rate and intensity of the reaction were increased. The formazan deposits measured 0.3 to 0.6 μ, and some were localized at the periphery of swollen globose mitochondria which measured 2 to 5 μ in diameter (Fig. 3). On the third day the number of swollen mitochondria was increased. On the fourth and fifth days the intensity of the reaction was decreased markedly, and rod-shaped mitochondria were absent (Fig. 4). This was most pronounced in the case of isocitric dehydrogenase. From the sixth through the ninth days the reaction was weak and diffuse, and fine intracellular localization of enzyme activity was no longer possible.

Alkaline Phosphatase. Strong activity was observed in the proximal and distal segments of the proximal convoluted tubules (Fig. 5). The reaction was strongest in the brush border, with a weaker, diffuse reaction in the remaining cytoplasm. No activity was seen in the tubules located in the medulla. After 5 days of vitamin D administration, a weaker reaction was noted. On the seventh day a very weak to negative reaction was observed around calcium deposits (Fig. 6). After 9 days of treatment, enzyme activity remained essentially unchanged, although the epithelium of dilated tubules frequently exhibited little or no activity. In no instance was a high alkaline phosphatase activity observed around calcific deposits.

Indoxyl Esterase. Nonspecific esterase activity was strongest in the proximal convoluted tubules (Fig. 7). The descending and ascending limbs of Henle's loop exhibited a weak activity, while the papilla contained only an occasional focus of weak activity. The enzyme was distributed diffusely throughout the tubular epithelial cytoplasm. On the fifth day of vitamin D administration, an increase in enzyme activity was seen in the proximal convoluted tubules and in the descending and ascending limbs. An occasional tubule exhibited an intense reaction which was localized both in the lining epithelium and in desquamated cells (Fig. 8). In many instances, intensely active amorphous material

corresponding to calcific casts was seen in the lumens of collecting ducts. Enzyme activity in the flattened epithelium lining dilated tubules was markedly decreased.

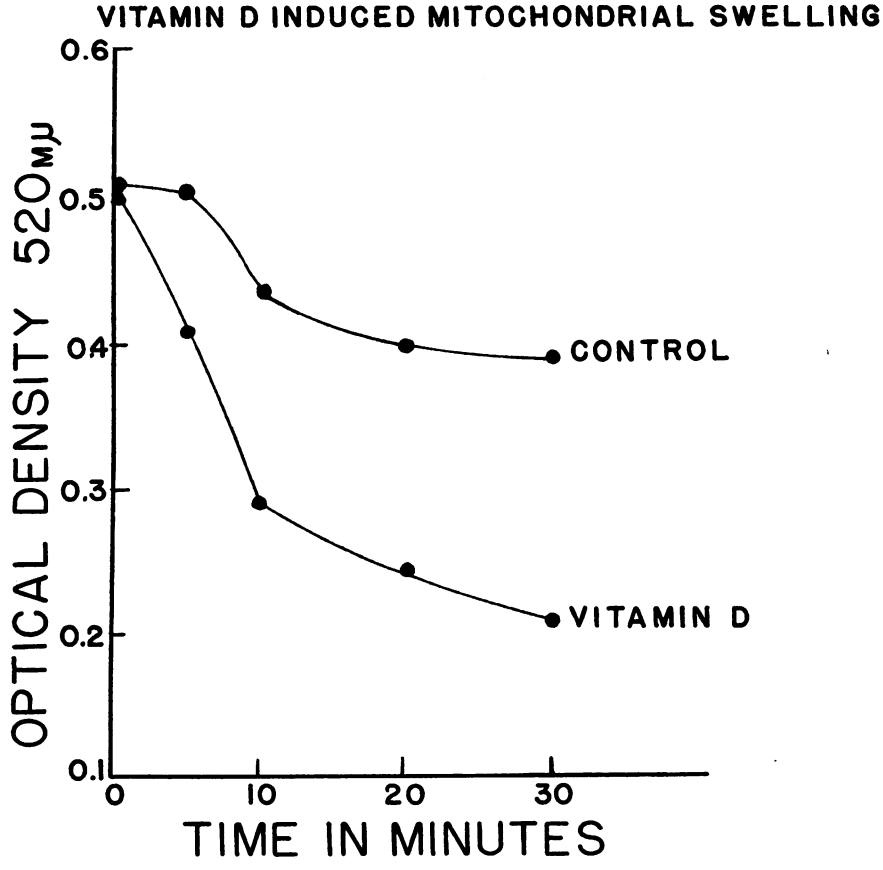
Treatment of renal sections with E-600 caused a striking inhibition of indoxyl esterase activity both in normal animals and in those receiving vitamin D. The proximal convoluted and distal tubules, the collecting ducts, and interstitial stromal cells of the cortex exhibited sites of resistant esterase activity. Following vitamin treatment, the activity of all these elements was increased. Desquamated cells showed the most intense E-600-resistant, cathepsinlike esterase activity.

Oil Red O. Neutral fat was demonstrable only in the renal papillas in normal rats. In one instance, positively stained droplets were observed in focal areas in the inner and middle zones of the cortex. Subsequent histologic examination showed these to represent sites of pyelonephritis. After 2 to 3 days of vitamin administration, oil red O-positive droplets, 2 to 5 μ in diameter, were observed in the proximal convoluted tubules; the formazan deposits were located at the periphery of the droplets (Fig. 9). From the fourth through the ninth days these droplets increased in size, often measuring 5 to 11 μ, and were found in larger numbers in most of the tubules.

Mitochondrial Staining. Mitochondria in the renal tubules of normal controls appeared as rodlets of varying lengths. They were most often oriented at right angles to the lumens (Fig. 10). Forty-eight to 96 hours after vitamin D administration, a diminution of rod-shaped mitochondria and the appearance of numerous spherical, swollen ones (1.5 to 2.7 μ in diameter) were noted (Fig. 11). Absolute counts of the number of damaged organelles per cell were not done. During the early stages of vitamin D treatment, these changes were present in small groups of proximal convoluted tubules, especially those in the inner cortex near the corticomedullary border. However, as treatment continued, more tubules were affected, including those in the middle and outer cortex.

Periodic Acid-Schiff Reaction (PAS). The proximal convoluted tubules were the most strongly PAS-positive structures observed in the normal kidney. The brush border exhibited an intense, diffuse staining, while the remaining cytoplasm contained moderate numbers of PAS-positive, diastase-resistant granules measuring 1 to 2 μ in diameter. After 4 days of vitamin D administration, staining of the brush border was reduced, and the PAS-positive cytoplasmic granules coalesced into 3 to 5 μ globules and rodlets. In many instances these appeared to be more numerous at the apical portion of the epithelium near the brush border. Many PAS-positive granules and globules were also noted in the lumens of these tubules (Fig. 12). From the fifth through the ninth days,

increasing numbers of cells in the descending and ascending limbs of Henle's loop exhibited an intense, diffuse, amorphous, nongranular, PAS-



TEXT-FIGURE 1. A comparison of spontaneous swelling of mitochondria in normal control rats and vitamin D-treated rats.

positive cytoplasmic staining. In many instances an intense staining was observed in the adjacent basement membrane and stroma (Fig. 13). Many dilated tubules showed weakly stained brush borders and contained strongly PAS-positive, homogeneous casts (Fig. 14). Calcific deposits and the tissue adjacent to them were also intensely PAS-positive (Fig. 15).

Millon and Sakaguchi Reactions. Cytoplasmic granules in the proximal convoluted tubules in vitamin D-treated animals showed positive reactions for tyrosine and arginine-containing proteins. They were identical in size, shape, and intracellular location to the PAS-positive granules. Homogeneous casts in the lumens of the proximal convolutions on the fifth day also exhibited positive reactions.

Results Obtained by Biochemical Techniques

Mitochondrial Swelling. In Text-figure 1 are shown comparisons of the rates of spontaneous and vitamin D-induced swelling of mitochon-

TABLE I

SERUM AND KIDNEY CALCIUM CONCENTRATION IN CONTROL AND VITAMIN D-TREATED RATS

Animals*	Days of treatment	Serum calcium, mg. per 100 ml.	Kidney calcium, mg. per 100 gm. of dry weight
Control	0	9.8–12.3	36–52
Treated	3	10.5–16	80–300
	6	13.5–20	1400–3120
	9	12.2–22	1440–3960

* Each group consisted of 9 animals.

dria. The rate was considerably greater in mitochondria isolated from vitamin D-treated rats.

Serum and Kidney Calcium. Serum calcium levels in control animals ranged from 9.8 to 12.3 mg. per 100 ml. After 3 days of vitamin D treatment, serum levels ranged from 10.5 to 16 mg., on the sixth day from 11.5 to 20 mg., and on the ninth day from 11.2 to 22 mg. per 100 ml. Kidney calcium levels in normal controls ranged from 36 to 52 mg. per 100 gm. of dry, fat-free tissue. After 3 days of vitamin D treatment, kidney calcium levels ranged from 80 to 300 mg., on the sixth day from 1,400 to 3,120 mg., and on the ninth day from 1,440 to 3,960 mg. per 100 gm. These results are summarized in Table I.

Kidney Citrate. The concentration of citrate in control rat kidneys ranged from 2 to 4 μg. per mg. of protein nitrogen. After 3 days of vitamin D treatment, the range increased from 12.5 to 20 μg. On the sixth day of treatment, the highest values were observed; these ranged from 33 to 52 μg. By the ninth day the citrate levels had fallen; they ranged from 8 to 29 μg. The observations are summarized in Text-figure 2.

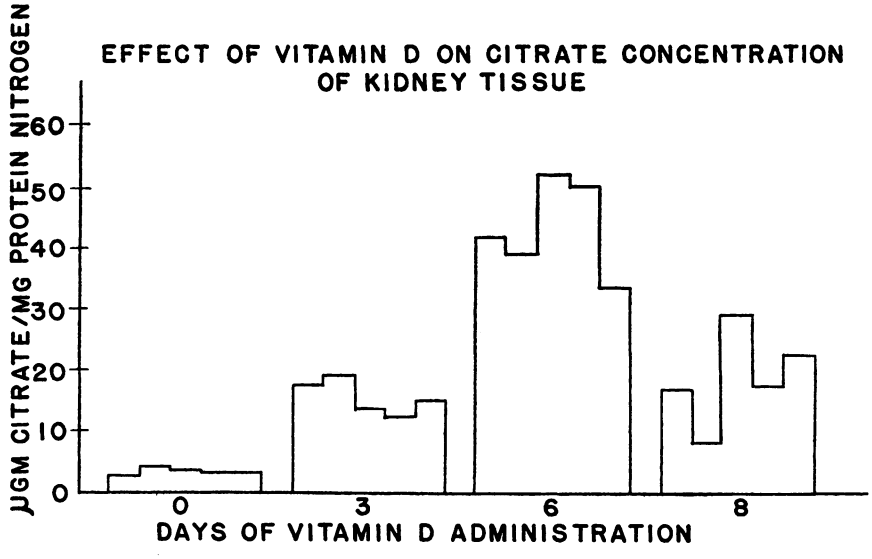
Cytopathologic Changes

Cytologic alterations in the tubular epithelium first became evident after 4 days of vitamin D administration. The lesion consisted of hydropic degeneration (Fig. 16) which was limited for the most part to the proximal convolutions. The distribution was patchy, involving groups of tubular cells at the corticomedullary junction and outer medulla. An occasional ascending limb of Henle's loop contained a finely granular basophilic substance in its lumen. On the fifth day numerous proximal convoluted tubules and both limbs of Henle's loop contained homo-

geneous eosinophilic casts with entrapped leukocytes and cellular detritus (Fig. 17). Some of the tubular cells showed an intense cytoplasmic basophilia, pyknotic nuclei, and contained a finely granular basophilic material (Fig. 18). In addition, several tubules contained amorphous, deeply basophilic, intracellular and intraluminal deposits, necrosis of tubular epithelium, and an occasional epithelial cell in mitosis. The basement membrane was extensively thickened and basophilic.

On the sixth and seventh days an increased number of tubules exhibited similar cytologic changes. The basement membranes of the proximal convolutions and Henle's loop showed even greater thickening and basophilia. In several instances large deposits of basophilic hyaline material were present between the basement membrane and the epithelial cells, displacing the latter into the lumen (Fig. 19); this was also seen in the calyces.

No new cytologic alterations were observed in the last two days of the experiment, although there was an increase in the number of affected tubules. Many tubules were dilated and exhibited atrophy of their lining cells (Fig. 20). The epithelium was flattened and contained a hyaline, eosinophilic cytoplasm. Some cells had desquamated into the lumen. A



TEXT-FIGURE 2. The effect of vitamin D on the citrate concentration of rat kidney tissue.

mild inflammatory reaction consisting wholly of histiocytes was seen around large calcific deposits. In no instance were cytologic changes observed in blood vessels or glomeruli.

Discussion

The pathogenesis of metastatic calcification has remained obscure since Virchow's classic description [24] of the lesions in 1854. The inability of many investigators to demonstrate definite evidence of cellular damage prior to the deposition of calcium salts has been largely responsible for the firmly rooted concept that metastatic calcification occurs in essentially normal tissues.[25] The present study has clearly shown that extensive morphologic and cytochemical damage at the mitochondrial level precedes calcification by a considerable period of time. This corroborates previous reports which have shown that mitochondria were among the most sensitive indicators of cell function and damage.[26–28]

The striking resemblance of the renal lesions produced by hypervitaminosis D and hyperparathyroidism led Anderson [29] to suggest that the renal lesions were the result of hypercalcemia, the only factor which was common to both conditions. The deleterious effects of calcium ions on mitochondrial structure and function have been demonstrated by various investigators.[30, 31] However, hypercalcemia may not be the only mechanism by which large doses of vitamin D exert a toxic effect on kidney epithelium. Recently de Luca, Reiser and Steenbock [32] showed that vitamin D added to a suspension of renal mitochondria was capable of inhibiting their metabolism.

It is now generally agreed that certain oxidative enzymes are associated exclusively with the mitochondria.[33–35] In addition, biochemical data indicate that the mitochondria probably represent a link between the energy-producing system of the cell and the conversion of energy to forms capable of driving the various synthetic and functional activities of the cell.[36, 37] Thus, injury at this level would be expected to result in a serious disturbance of these processes.

The marked reduction of DPN and TPN diaphorase activity indicates that the activities of all the coenzyme I and II linked dehydrogenases are impaired early in vitamin D-induced nephrocalcinosis. The decrease in enzyme activity and the diffusion of DPN-linked isocitric dehydrogenase from swollen mitochondria are no doubt responsible for the accumulation of citrate observed in renal tissue. This is in keeping with current views that the adverse alterations in biochemical function accompanying mitochondrial damage are due to the loss of soluble enzymes, coenzymes and cofactors by passage across altered mitochondrial membranes.[38, 39] This initial change in cell metabolism precedes any microscopically visible alteration in the tubular epithelium and may represent a "biochemical lesion" as described by Gravilescu and Peters.[40] Inasmuch as citrate turnover is highest in the kidney,[41] ac-

cumulation of this metabolite may not be a specific vitamin D effect but simply the result of damage to renal epithelium.

Since citrate forms a soluble, non-ionized complex with calcium, the high citrate levels in kidney tissue probably influence the deposition of calcium to a considerable degree. Although high concentrations of citrate inhibit the calcification of rachitic rat bone slices *in vitro*,[42] Kuyper[43] showed that small amounts of citrate in solution are completely co-precipitated with calcium phosphate and carbonate upon the addition of these ions. Therefore, Hass's view[44] that diffusible, non-ionized, organic calcium compounds secondarily attract phosphate ions and result in metastatic calcification may be correct.

The hydropic changes in tubular epithelium which became evident two days after the onset of mitochondrial swelling and reduced diaphorase activity suggest a relation between the content of intracellular water and mitochondrial integrity. This is in accord with the observations of Macfarlane and Spencer[45] and Bartley, Davies and Krebs[46] that the active transport of water and electrolytes in cells is a function of the mitochondria. Tissue changes similar to hydropic degeneration were observed by Robinson[47] in kidney slices in which respiration was inhibited by cyanide or uncoupled by 2,4 dinitrophenol. Thus the water and electrolyte balance of tissues depends upon their metabolism in addition to the osmotic pressure of extracellular fluid.

The simultaneous appearance of intracellular neutral fat droplets and the disappearance of enzymatically active mitochondria suggest a relationship between the two. Although this has been suggested by histologists for some time, confirmatory evidence had only recently been obtained. Christie and Judah[48] have reported that 36 hours after carbon tetrachloride administration, numerous neutral, fat-positive globules were isolated from the mitochondrial fraction of rat liver. These exhibited succinoxidase activity and contained ribonucleic acid, two characteristics of mitochondria. According to Dixon,[49] the appearance of visible fat droplets may be due to the conversion of invisible micellar complex mitochondrial lipid following cell injury. However, this problem is by no means settled, as evidenced by the recent report of Recknagel and Anthony[50] that lipid accumulation may appear considerably earlier than mitochondrial degeneration. They suggest that the two processes may occur independently of each other.

During the early stages of swelling, many mitochondria resemble the so-called "target forms" described by Harman and Feigelson.[51] The eccentric peripheral localization of enzyme activity in the globose organelles is similar to the grouping and displacement of cristae observed with the electron microscope in swollen mitochondria by Hogeboom, Kuff and

Schneider.[52] However, their identity remains to be established with certainty.

Shortly after the onset of mitochondrial swelling, the PAS-positive, diastase-resistant granules in the cytoplasm of the proximal convoluted tubule cells increased in number and size. As damage progressed to the point of reduced enzyme activity, the number of granules increased, filling the cytoplasm and in some instances the tubule lumens. Although their origin is at present unknown, these PAS-positive protein granules may represent mucoprotein mobilized from the ground substance of bone by massive doses of vitamin D, as postulated by Eisenstein and Groff.[53] In addition, since the resorption of protein by the cells of the proximal convoluted tubules appears to be intimately associated with mitochondria,[54–57] and probably depends on their function, the droplets may also be the result of impaired protein resorption by the damaged organelles. The high intracellular concentration of mucoprotein in the various components of the nephron may determine in part the deposition of calcium in these sites. The affinity of various components of the ground substance for calcium has been shown repeatedly.[58–61]

Although the role of alkaline phosphatase in normal calcification has been defined, its relation to pathologic calcification is by no means settled.[62] Gomori [63] found that calcification in bone tumors and granulation tissue was always associated with alkaline phosphatase activity; however, he was not able to show such an association in calcification of the aorta. Our results indicate that calcification can occur at sites devoid of alkaline phosphatase activity. This is in agreement with Waldman [64] and McLean's [65] observations that calcification can occur in cartilage in which enzyme activity has been inhibited either by heat or heavy metal treatment.

Increased activity of nonspecific esterase and cathepsin was found in the proximal convoluted tubules shortly after the onset of hydropic degeneration. The highest activity was localized in desquamated epithelium and in amorphous necrotic material in tubular casts. These changes represent the augmented activity of proteolytic enzyme systems characteristic of cell death.

The nephropathy induced by hypervitaminosis D is characterized by a sequence of cytochemical and morphologic changes which eventuate in tubular necrosis and calcium deposition. Our results suggest the following mode of pathogenesis: Large doses of vitamin D result in a mobilization of both calcium and mucoprotein from the ground substance of bone. The hypercalcemia causes cell injury at the mitochondrial level and is identical with cellular sites of calcification seen in the later stages of this lesion. Mitochondrial swelling results in a loss of isocitric dehydrogenase

activity due to diffusion of the enzyme, which in turn causes an accumulation of citrate in the renal epithelium. The damaged cells of the proximal convolutions are unable to resorb proteins, and these substances accumulate in the kidney cells and eventually in the tubule lumens. The affinity of mucoproteins for calcium ions coupled with the increased tissue concentration of citrate capable of complexing calcium may provide a mechanism by which phosphate and carbonate ions are secondarily attracted to form calcific deposits.

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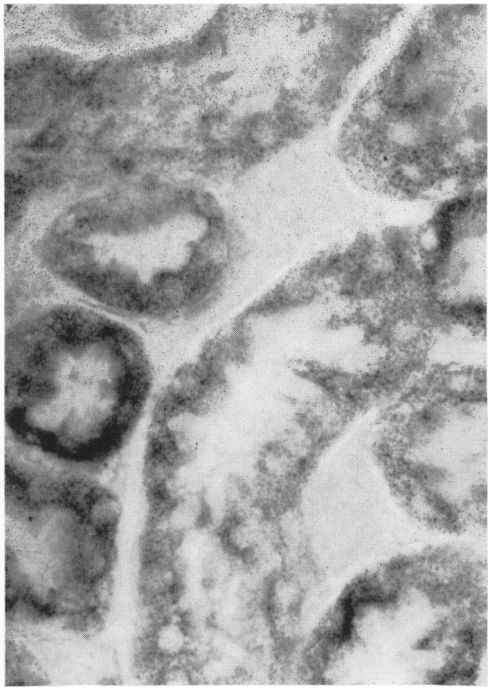
LEGENDS FOR FIGURES

FIG. 1. Normal rat kidney. Sites of DPN diaphorase activity appear in the proximal convoluted tubules. DPN diaphorase reaction. × 650.

FIG. 2. Rat sacrificed after 2 days of vitamin D treatment. There is variation in DPN diaphorase activity. Swollen mitochondria are present in the tubule in center of the photograph. Rod-shaped mitochondria are manifest in the surrounding tubules. DPN diaphorase reaction. × 650.

FIG. 3. A higher magnification of the damaged tubule shown in Figure 2. Formazan deposits are localized at the periphery of swollen mitochondria. DPN diaphorase reaction. × 1450.

FIG. 4. Rat sacrificed after 4 days of vitamin D treatment. There are numerous swollen mitochondria and a complete absence of rod-shaped mitochondria. The diffuse nature of the reaction is probably due to enzyme diffusion from damaged organelles. Isocitric dehydrogenase reaction. × 650.



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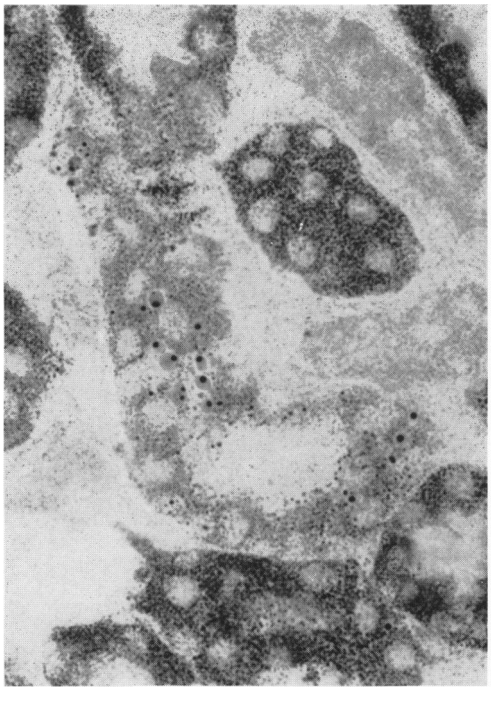

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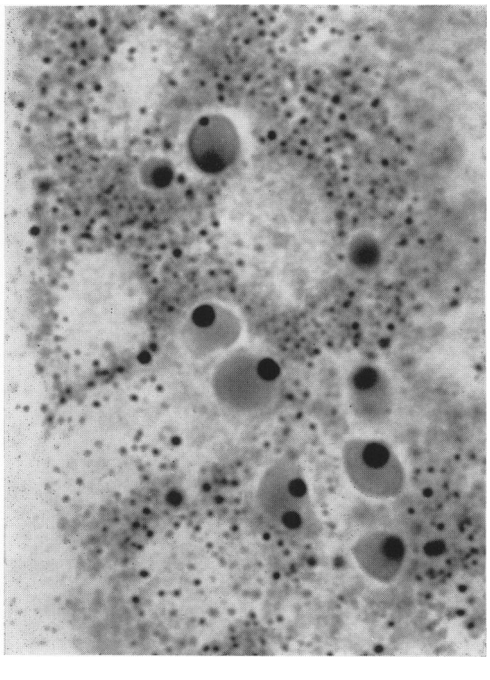

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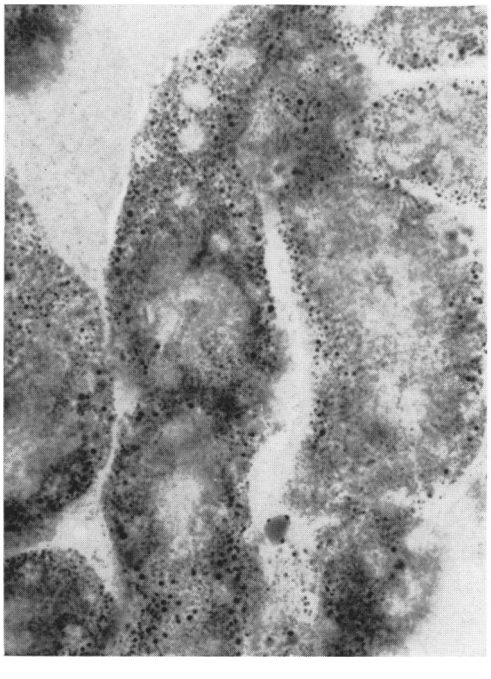

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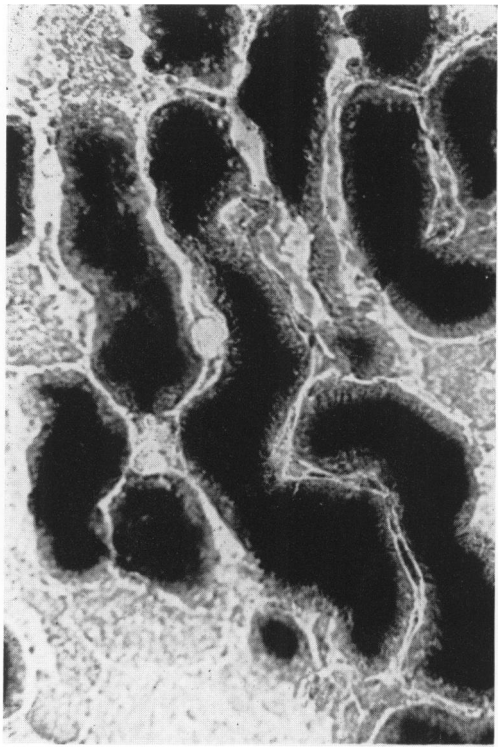
FIG. 5. Normal rat kidney. Alkaline phosphatase activity in the proximal convoluted tubules is localized in the brush border. Alkaline phosphatase reaction. × 650.

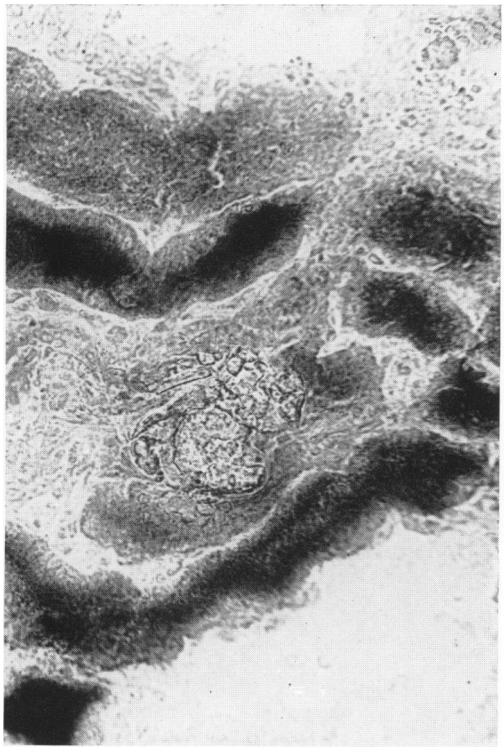
FIG. 6. Rat sacrificed after 7 days of vitamin D treatment. A very weak alkaline phosphatase activity is present around an intratubular calcific deposit. Alkaline phosphatase reaction. × 650.

FIG. 7. Normal rat kidney. Indoxyl esterase activity appears in the proximal convoluted tubules. Indoxyl esterase reaction. × 650.

FIG. 8. Rat sacrificed after 5 days of vitamin D treatment. Note the intense reaction in the desquamated cells and debris in the tubule lumens. Indoxyl esterase reaction. × 750.

5





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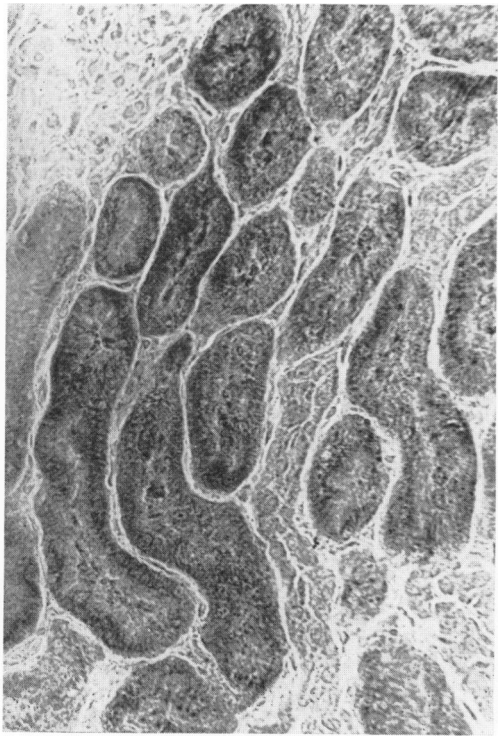

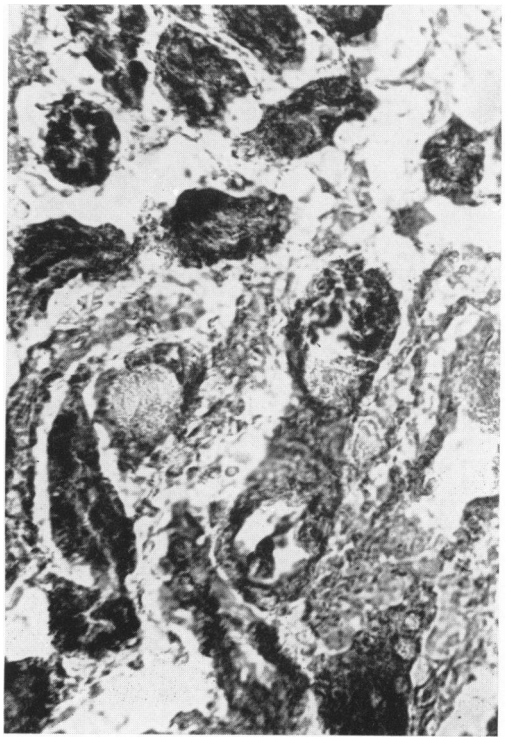

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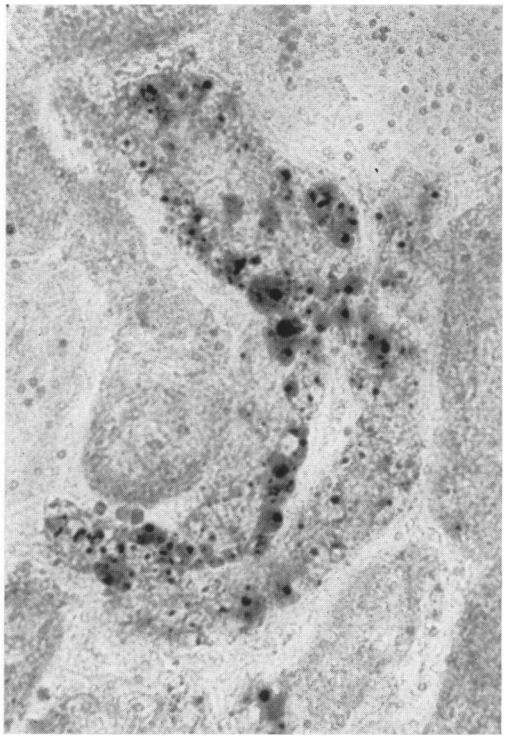
FIG. 9. Rat sacrificed after 3 days of vitamin D treatment. The swollen mitochondria exhibit an intense oil red O staining. DPN diaphorase reaction and oil red O stain. × 750.

FIG. 10. Normal rat kidney. Numerous rod-shaped mitochondria are oriented at right angles to the tubule lumens. Heidenhain's iron hematoxylin stain. × 600.

FIG. 11. Rat sacrificed after 3 days of vitamin D treatment. There are swollen spherical mitochondria and a few remaining rod-shaped forms. Heidenhain's iron hematoxylin stain. × 650.

FIG. 12. Rat sacrificed after 4 days of vitamin D treatment. Epithelium of the proximal convoluted tubules contains large numbers of PAS-positive granules which fill the cells and tubule lumens. Periodic acid-Schiff (PAS) reaction. × 650.



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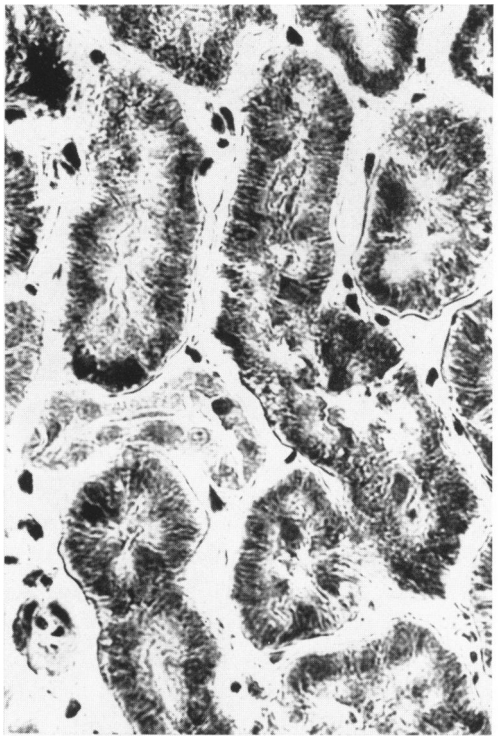

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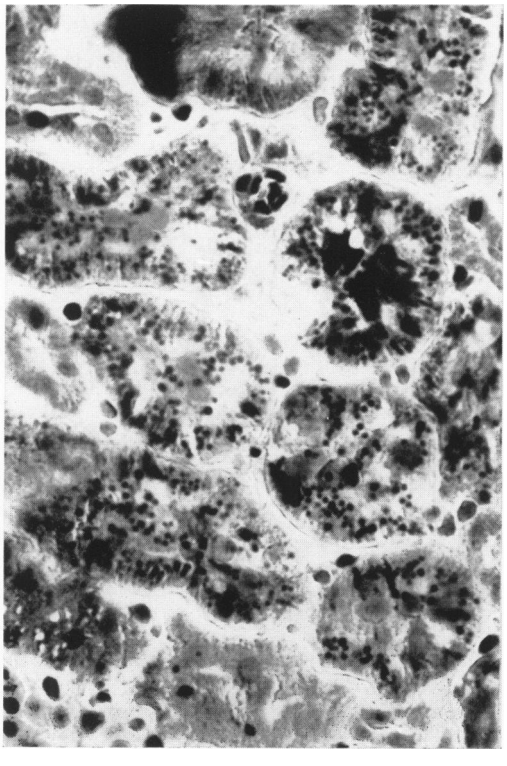

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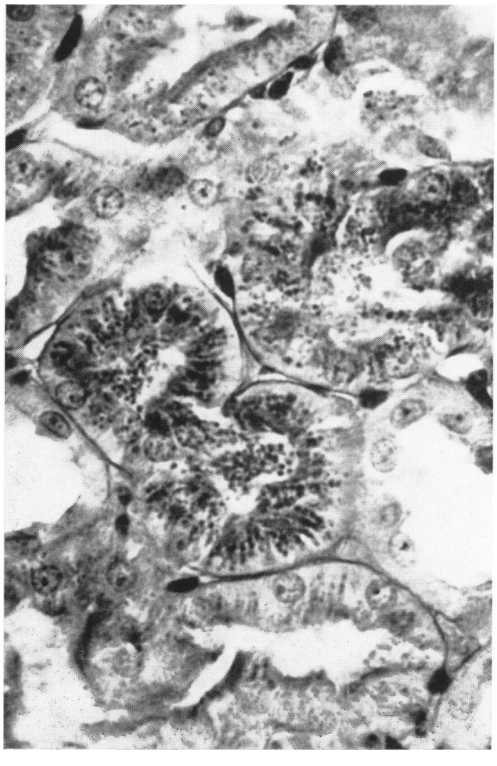

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FIG. 13. Rat sacrificed after 5 days of vitamin D treatment. An intense PAS reaction is present in the basement membrane and adjacent stroma of a dilated proximal convoluted tubule. PAS reaction. × 300.

FIG. 14. Rat sacrificed after 6 days of vitamin D treatment. Intensely PAS-positive protein casts appear within the renal tubules. PAS reaction. × 260.

FIG. 15. Rat sacrificed after 7 days of vitamin treatment. Small, spherical, calcific deposits and the basement membrane are PAS-positive. PAS reaction. × 350.

FIG. 16. Rat sacrificed after 4 days of vitamin D treatment. A group of proximal convoluted tubular cells exhibit hydropic degeneration. Hematoxylin and eosin stain. × 600.

The sections illustrated in Figures 17 to 20 were stained with hematoxylin and eosin.

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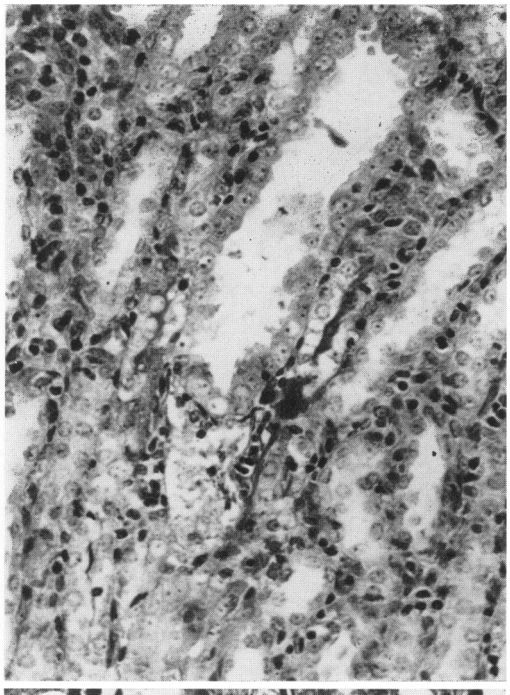

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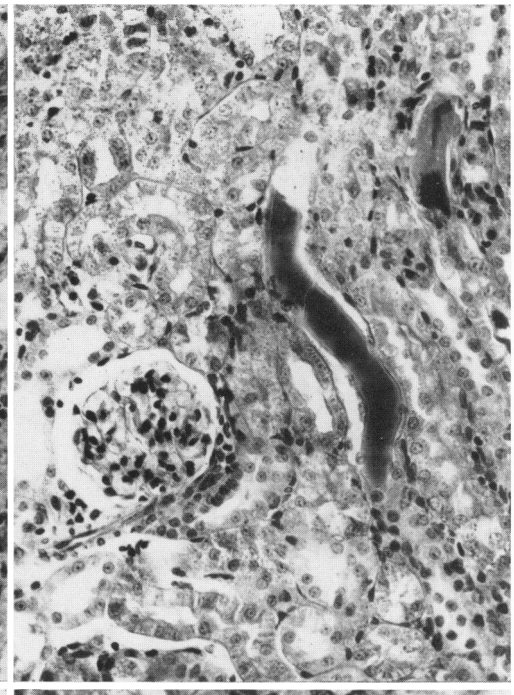

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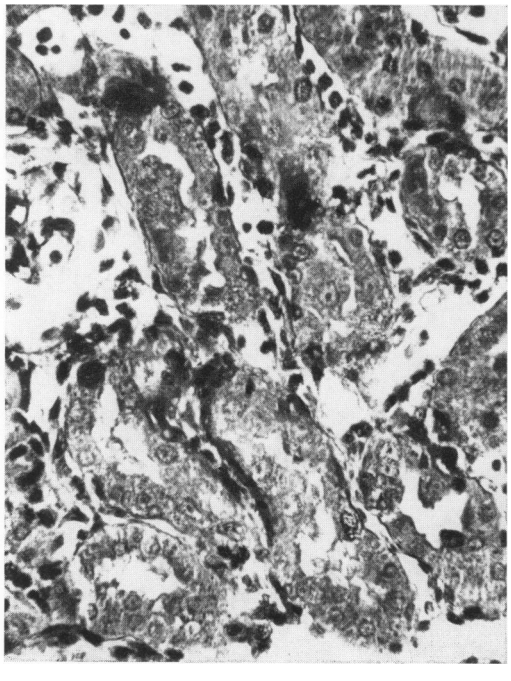

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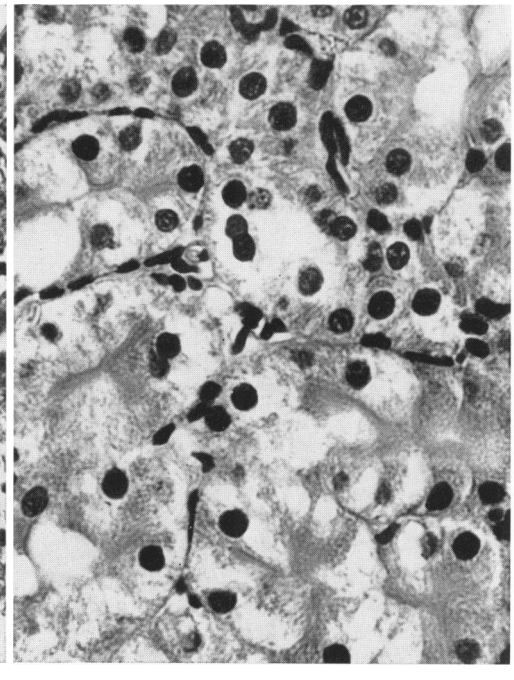


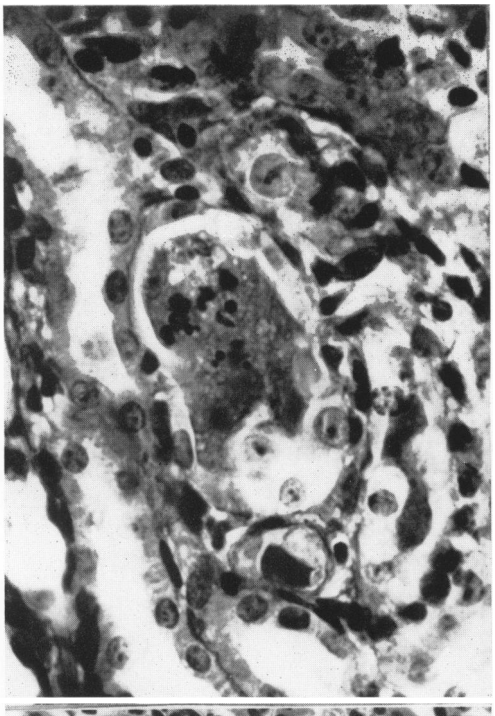
FIG. 17. Rat sacrificed after 5 days of vitamin D treatment. A protein cellular cast is manifest in the descending limb of Henle's loop. × 650.

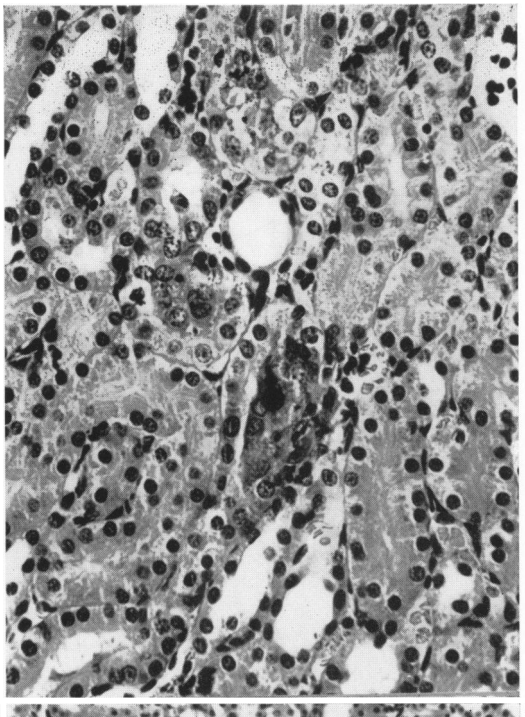
FIG. 18. Rat sacrificed after 5 days of vitamin D treatment. Tubular epithelium exhibits intense cytoplasmic basophilia and focal necrosis. × 400.

FIG. 19. Rat sacrificed after 6 days of vitamin D treatment. There are degeneration and calcification of the basement membrane with displacement of the tubular epithelium into the lumen. × 400.

FIG. 20. Rat sacrificed after 8 days of vitamin D treatment. Many dilated tubules with pressure atrophy of the tubular epithelium are evident. Note the numerous protein casts. × 150.

17





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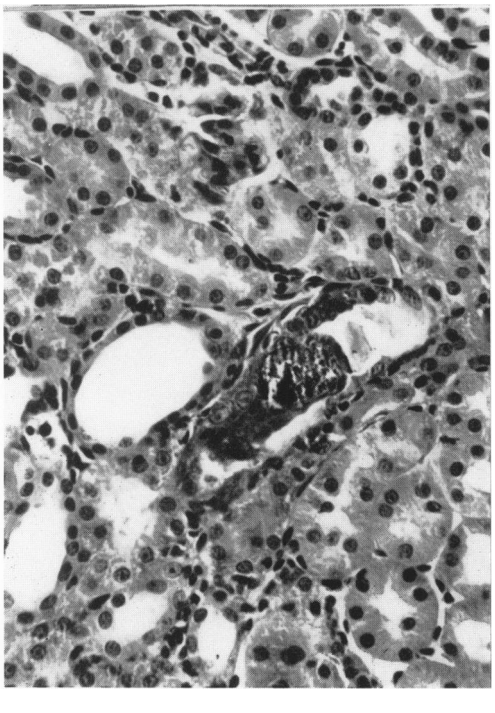

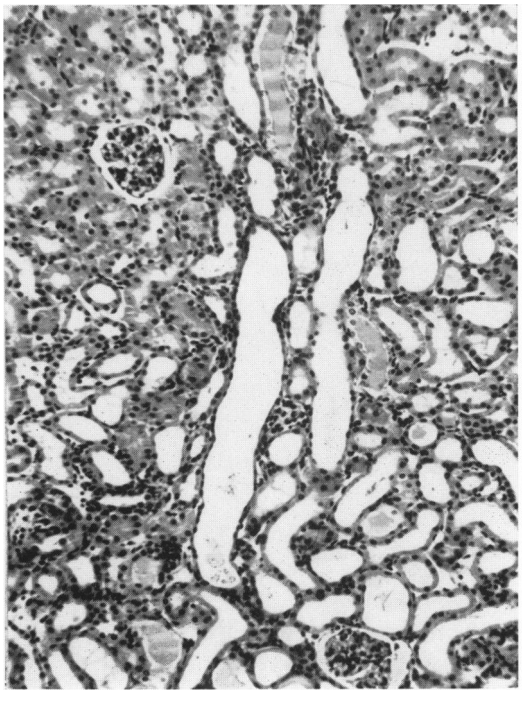

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