

# A STUDY OF METASTATIC RENAL CALCIFICATION AT THE CELLULAR LEVEL

FILIBERTO GIACOMELLI, M.D., DAVID SPIRO, M.D., and  
JOSEPH WIENER, M.D.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University,  
New York

## ABSTRACT

Experimental metastatic calcification in the proximal convoluted tubules of rat kidney, produced by large doses of vitamin D, has been studied with a variety of techniques. These techniques include the examination of thin sections of Araldite-embedded material under the electron microscope, selected area electron diffraction, and several histochemical methods. Two types of mineral are found in relation to the proximal convoluted tubule. The first form consists of aggregates of elongated crystals within cytoplasmic vacuoles of the proximal tubular cells. The dimensions of these crystals are consistent with those of hydroxyapatite. The other type of mineral deposit is found in and adjacent to the extracellular phase of the basal infoldings of these tubules. The latter deposits are made up of smaller crystals arranged in layers. These crystals could not be definitely identified by means of selected area electron diffraction. The observations are discussed in relation to calcium transport by the proximal convoluted tubule and also in terms of mechanisms of pathological calcification.

## INTRODUCTION

The administration of large doses of vitamin D produces metastatic calcification of various portions of the nephron (1-13). In acute poisoning with vitamin D, the calcium deposits occur primarily in the proximal convoluted tubules (12, 13). More prolonged administration of this drug results in calcific lesions involving other portions of the kidney (1-11). However, there is relatively little information concerning this process at the cellular level, despite numerous histological and pathophysiological studies of the hypervitaminotic D state.

The purpose of this study is to define, by ultrastructural techniques, the localization of the mineral following acute poisoning with vitamin D, the nature and crystalline habit of this material, and

the significance of the deposits in relation to calcium transport by the proximal convoluted tubular cells.

## MATERIALS AND METHODS

Thirty-two Holtzman albino rats, weighing 150 gm, were fed Purina rat chow *ad libitum*. Sixteen of these animals also received crystalline vitamin D<sub>2</sub> (calciferol, Philips Roxane Laboratories, Columbus, Ohio) diluted with purified sesame oil, by stomach tube, in a dose of 30,000 units per 100 gm weight daily (13). The animals were weighed daily and sacrificed after 12 hours and 2, 4, 6, 8, 10, 12, and 14 days of vitamin administration. Two animals were sacrificed at each of these periods. Two control animals, which did not receive the vitamin supplement, were also sacrificed at each of these intervals. Both the inner and outer

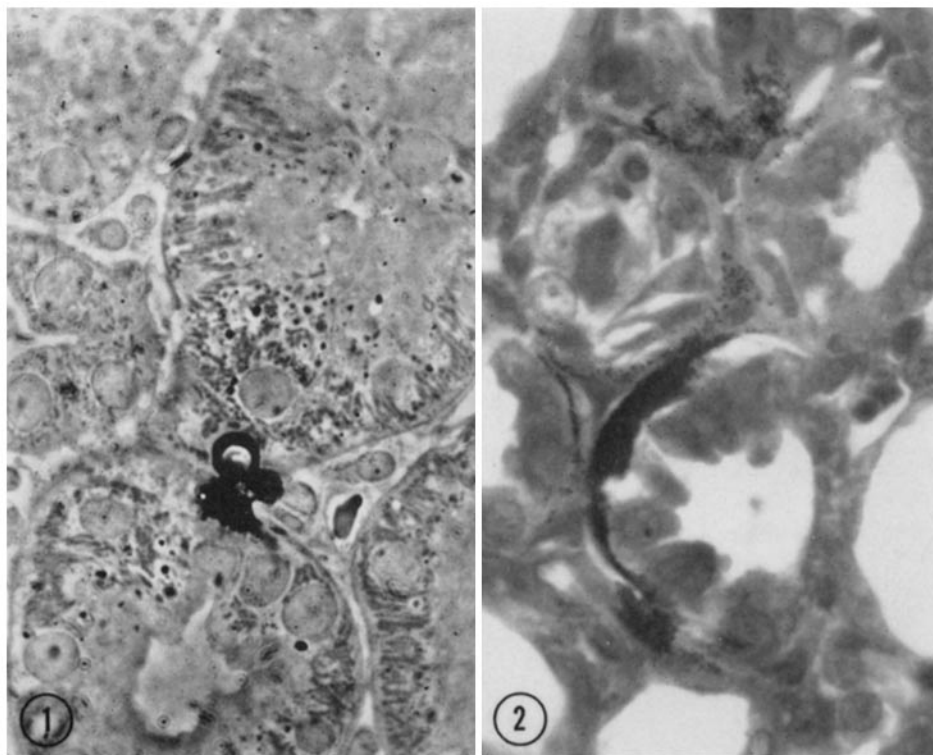


FIGURE 1 This phase contrast photomicrograph shows a mineral deposit at the basal aspects of two adjacent proximal convoluted tubules. In addition, numerous dense granules are seen within some of the tubular cells.  $\times 1000$ .

FIGURE 2 This paraffin-embedded section exhibits positive von Kossa staining of the mineral which appears to extend along the basement membrane of a tubule. Numerous von Kossa-staining cytoplasmic granules are also present.  $\times 1000$ .

renal cortical tissues from both the control and vitamin D-fed animals were excised, immediately fixed for  $1\frac{1}{2}$  hours in 1 per cent buffered (Veronal-acetate) osmium tetroxide at pH 7.4 with added sucrose at  $5^{\circ}\text{C}$ , dehydrated in acetone, and embedded in araldite (14).

Thick unstained sections, as well as thick sections stained with the periodic acid-Schiff (PAS) reagent, of this Araldite-embedded material, were examined

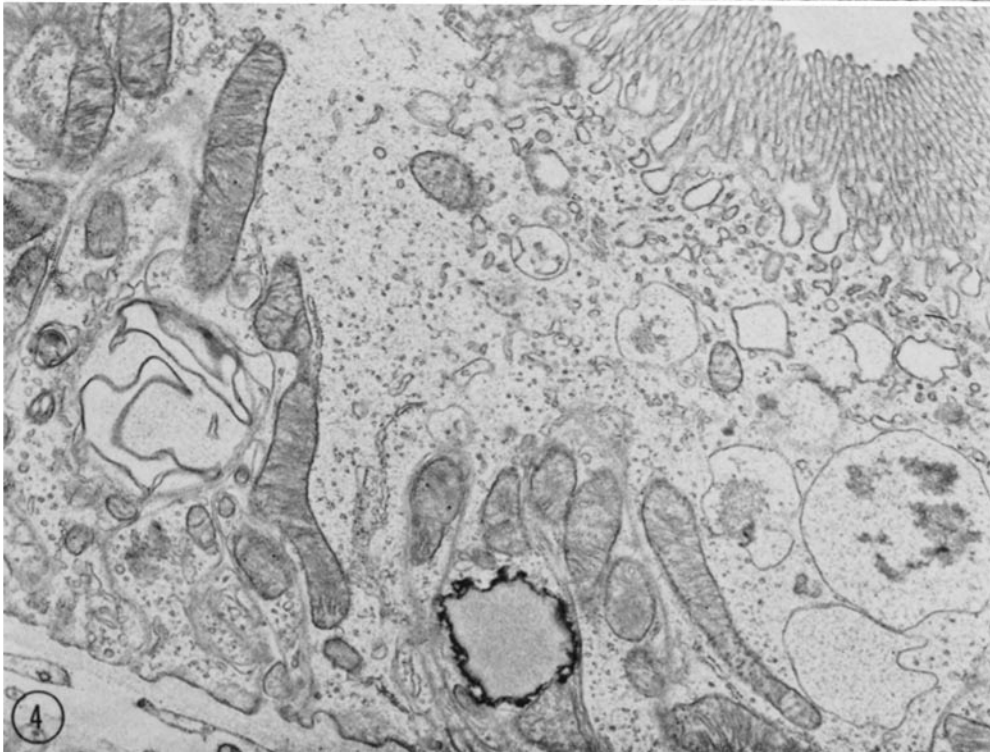
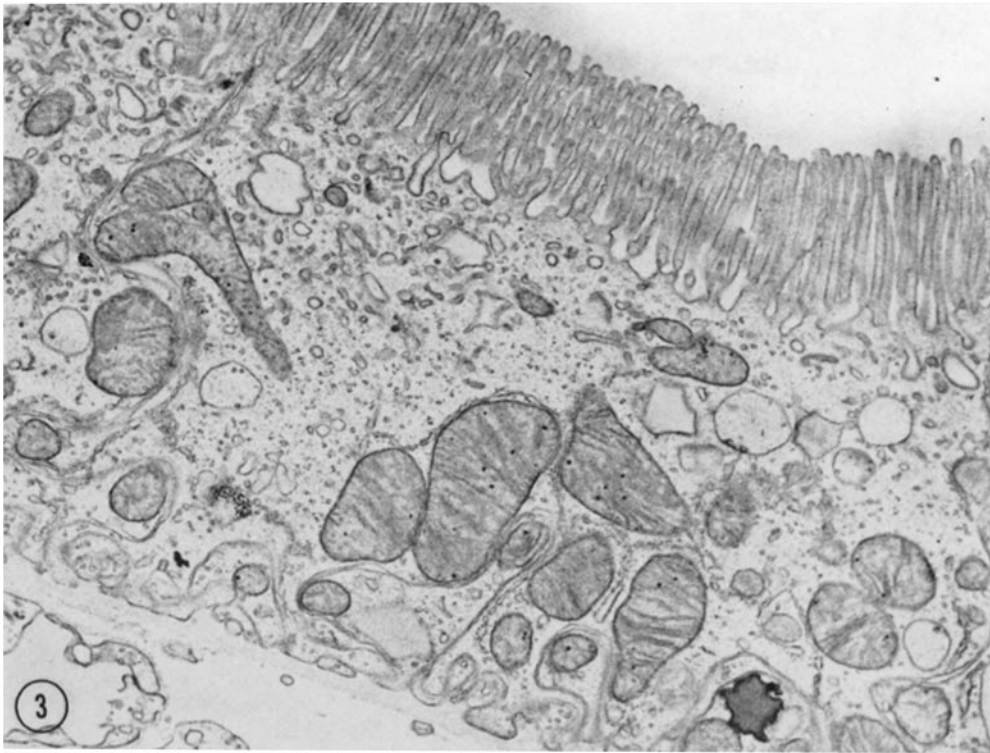
by phase and ordinary light microscopy. In addition, renal tissue was fixed in 10 per cent neutral phosphate-buffered formalin and embedded in paraffin. These sections were stained with hematoxylin and eosin and alizarin red S (15), and by the von Kossa method (16). A microchemical method for determining the presence of carbonates was also employed (17).

Thin sections were examined with an RCA EMU-

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FIGURE 3 This electron micrograph reveals flattening of the cells of the distal segment of a proximal convoluted tubule. Numerous vesicles, which are present in the luminal and midportions of the tubule, appear to originate as invaginations of the apical cell surface membrane. Note the lipid droplet in the basal portion of the tubule.  $\times 13,000$ .

FIGURE 4 Figs. 4 to 7 are from the proximal segment of the proximal tubule. Several intracytoplasmic vacuoles contain electron-opaque material. A large basally located vacuole on the left exhibits internal membranous structures.  $\times 12,000$ .



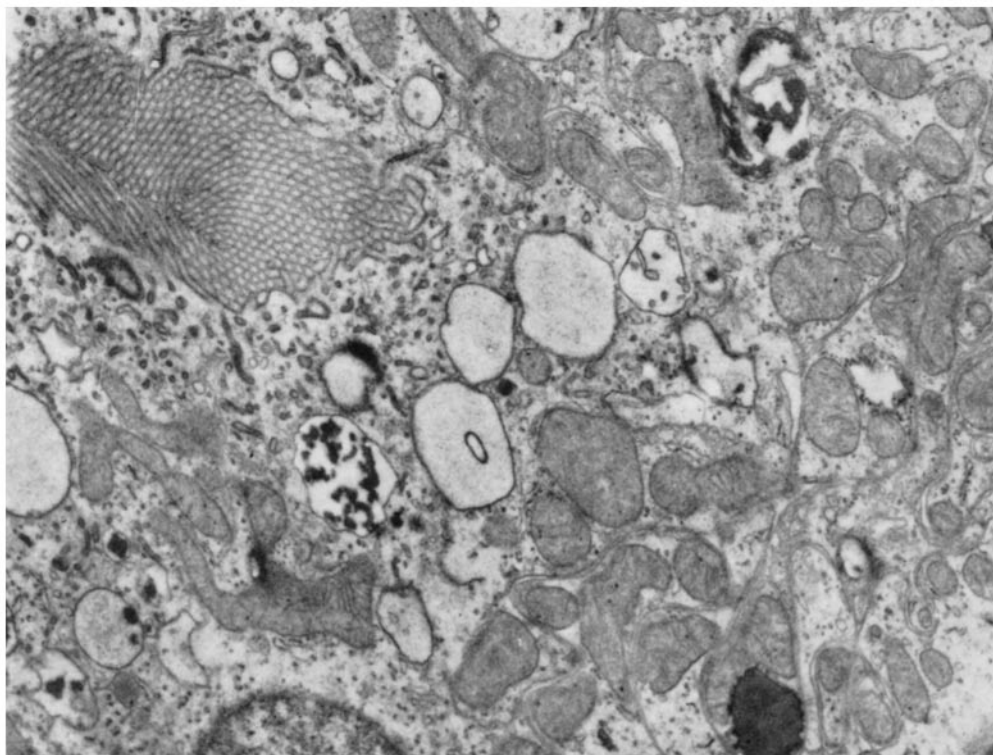


FIGURE 5 Deposits of very electron-opaque material are present within vacuoles.  $\times 12,000$ .

3F and a Siemens Elmiskop I. The thin sections were not stained, since it has been previously observed that electron stains dissolve tissue calcium (18). Selected area electron diffraction patterns of the calcific deposits were obtained from 1 micron thick Araldite-embedded sections with the Siemens Elmiskop I at 100 kv. Similar electron diffraction patterns from an evaporated gold film were utilized for calibration purposes.

#### RESULTS

The earliest changes observed by phase microscopy occur after 8 days of vitamin excess and consist of numerous dense granules within the cytoplasm of proximal convoluted tubular cells (Fig. 1). During the course of the experiment larger deposits appear and often extend along the basement membranes of the tubules (Fig. 1) and occasionally extend into the lumina of these tubules. The hardness of the

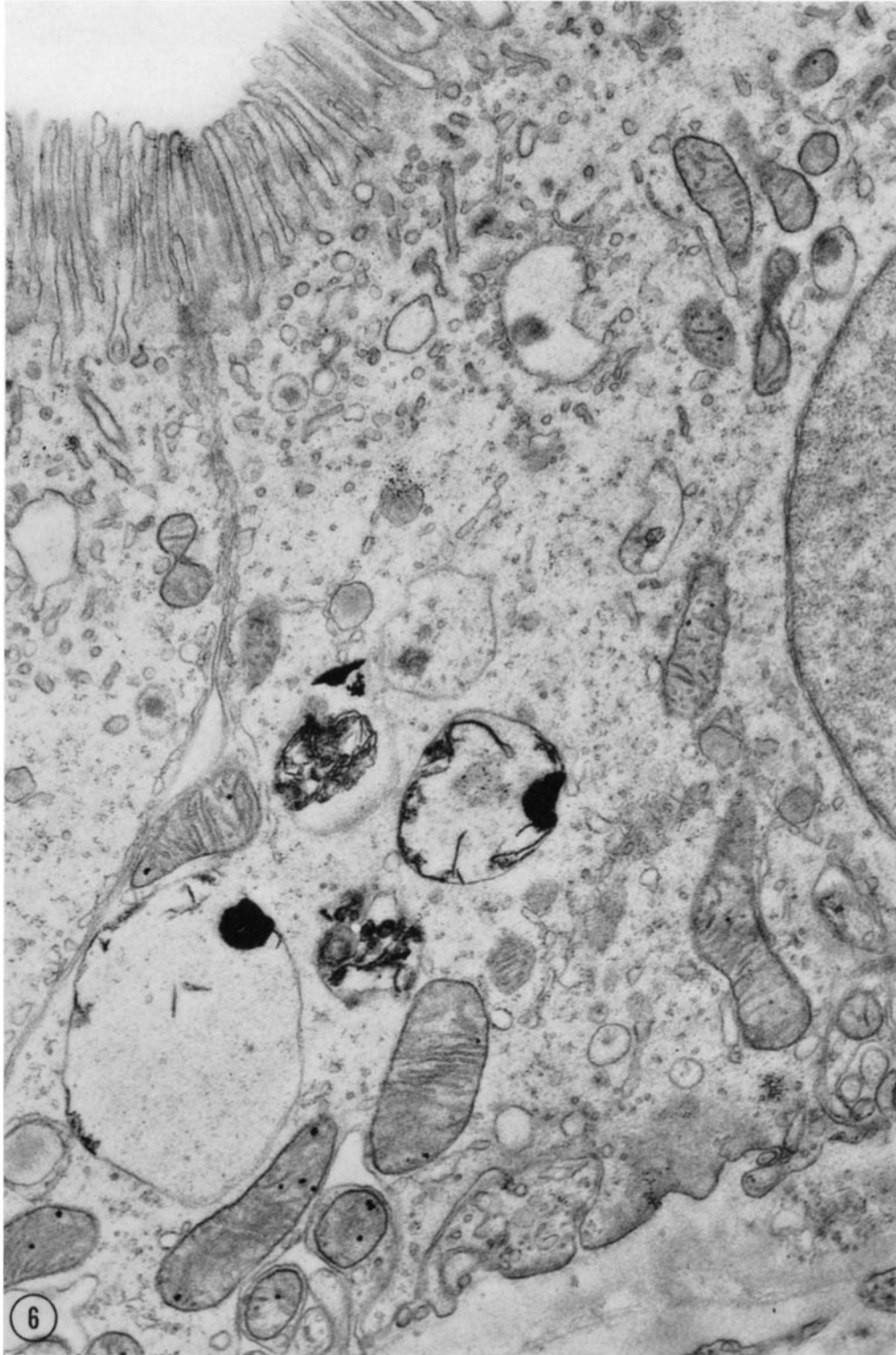
deposits results in frequent artifactual tears of the tissues in both thick and thin sections. However, in all instances the material is adequate for both light and electron microscopy. The intracellular granules as well as the larger aggregates stain positively with both the PAS and von Kossa stains (Fig. 2), and show reddish orange lakes with alizarin red S. Microchemical procedures for carbonates are negative (17).

The fine structural features of the proximal and distal segments of the proximal tubules of the control animals are similar to those described by Rhodin in the mouse (19) and Caulfield and Trump in the rat (20).

The earliest changes in the vitamin D-fed rats, as seen by electron microscopy, consist of flattening of the cells of the proximal convoluted tubules and dilatation of the lumina of the tubules (Fig. 3).

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FIGURE 6 Large vacuoles containing amorphous electron-opaque material and what appear to be either elongated crystals or membranes are shown.  $\times 18,000$ .



These tubular cells contain cytoplasmic vesicles and vacuoles which progressively increase in number and size during the course of the experiment and measure up to 2 to 3 microns in diameter. The vacuoles are present throughout the cells, are most numerous in the luminal and midportions of the tubule, and tend to be larger in the basal portions of the tubule (Fig. 4). The vacuoles are initially devoid of electron-opaque contents and are delimited from the cytoplasm by a single (unit) membrane (21) which in the apical portions is often continuous with the cell surface membranes. This indicates that these organelles are probably pinocytotic in nature (Figs. 3 and 4). After 6 days many of the vacuoles contain deposits of very high electron opacity (Figs. 5 and 6) as well as arrays of membranes exhibiting various configurations (Fig. 4). After 8 days some vacuoles contain aggregates of crystals (Figs. 6 to 9), the individual crystals usually measuring from 400 to 800 Å in length and about 50 Å in the other dimension (Figs. 8 and 9). Occasionally the crystals are over 1000 Å in length (Fig. 6). It was impossible to determine the precise nature of this material by selected area diffraction studies, despite numerous attempts utilizing thick sections. The vacuoles containing crystals correspond with the dense cytoplasmic granules seen with light microscopy (Figs. 1 and 2). These alterations involve both the proximal and distal segments of the proximal tubules, but are somewhat more prominent in the proximal segment. Additional cytological changes consist of increased numbers of subnuclear lipid droplets (Fig. 10). Mitochondria and other cellular organelles are not structurally altered.

During the 2nd week of the experiment another and more frequent form of deposition is observed in the interstitial tissue near the basal aspect of the proximal convoluted tubules. These deposits, corresponding to the larger aggregates seen under the light microscope (Figs. 1 and 2), consist of approximately cuboidal-dense particles measuring about 50 Å which are located in the extracellular phase of the basal infoldings (Figs. 11 to 13). Towards the end of the experiment larger aggregates measuring

many microns in diameter are noted. The larger extracellular masses are composed of similar particles organized in a definite pattern of concentric layers and sometimes extend from the region of the basal infoldings along the plane of the tubular basement membrane for a considerable distance (Fig. 14). These lamellated structures in greatly expanded basal infoldings may also extend from the basement membrane to the luminal aspect of the cell (Fig. 15). Occasionally, elongated crystals, similar to those observed within the cytoplasmic vacuoles, are seen in the extracellular foci of calcification (Fig. 16). Selected area electron diffraction patterns were obtained from thick sections containing the extracellular mineral. The diffraction patterns are indicative of an oriented, well crystallized phase and show six diffraction maxima. These reflections or d spacings are 6.2, 3.1, 2.89, 2.13, 1.84, and 1.68 Å. This crystalline material cannot be identified from the diffraction data available.

The pathologic alterations described are limited to the proximal portion of the nephron. The distal convoluted and collecting systems reveal no significant alterations by either light or electron microscopy.

#### DISCUSSION

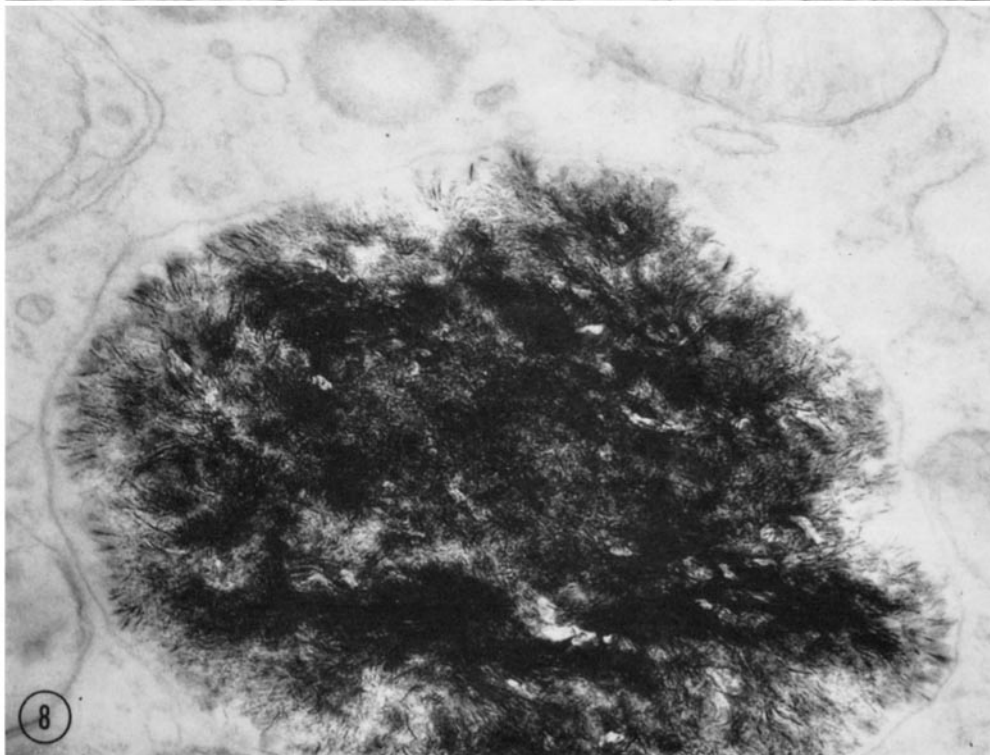
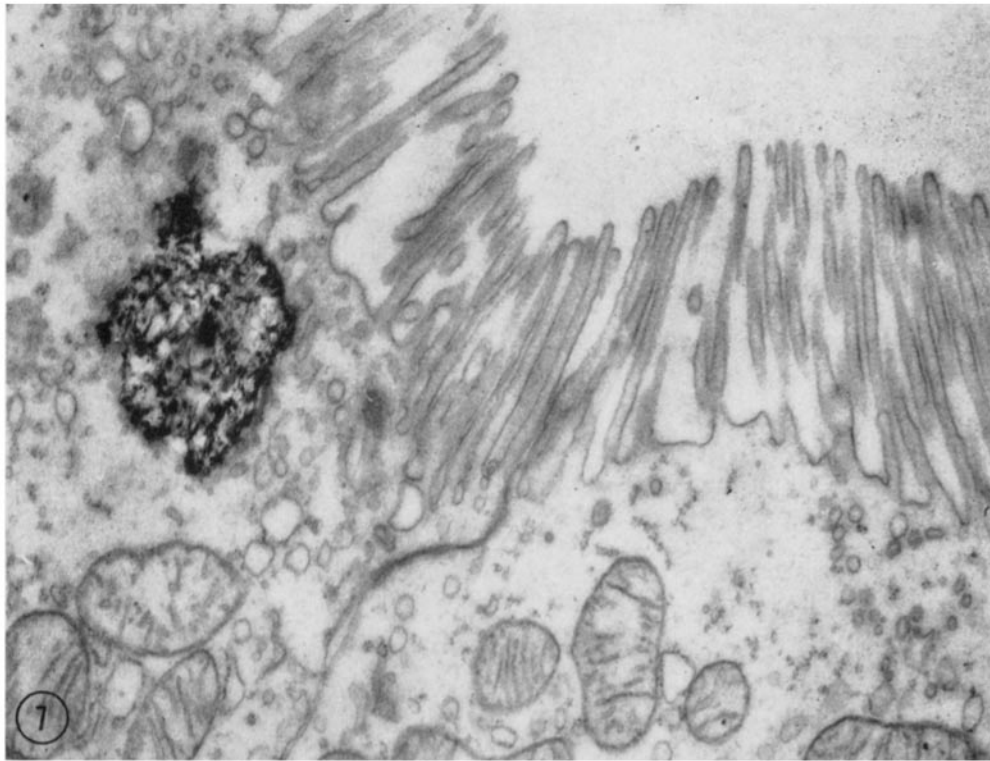
The administration of massive doses of vitamin D to rats over a relatively short period of time results in metastatic renal calcification which is primarily localized to the cortex (12, 13). Large amounts of vitamin D enhance the intestinal absorption of calcium (22). Another effect of this hypervitaminotic state is the dissolution of formed bone (13, 23). The fact that in this condition hypercalcemia and hypercalciuria antedate histologic evidence of renal damage, even in the presence of a calcium-deficient diet (24), suggests that metastatic renal calcification is secondary to the action of vitamin D on the skeleton (13, 23). The situation is further complicated by evidence which indicates that excessive doses of vitamin D may result in a parathormone-like effect on the kidney with respect to phosphate reabsorption (25).

Regardless of the relative importance of these

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FIGURE 7 An apical cytoplasmic vacuole contains numerous crystalline aggregates.  $\times 21,000$ .

FIGURE 8 This vacuole in a cell of a proximal convoluted tubule is completely filled with elongated crystals.  $\times 36,000$ .



various effects of vitamin D, two types of mineral deposition related to the proximal convoluted tubules are noted. The first form consists of mineral in cytoplasmic vacuoles of the cells of the proximal convoluted tubules. The second form is represented by extracellular mineral deposits which are found in the basal infoldings of these tubular cells. Both the intracellular and extracellular deposits exhibit reddish orange lakes when stained with alizarin red S and also give positive von Kossa reactions. Similar deposits examined under the electron microscope consist in part of very electron-opaque elongated crystals resembling hydroxyapatite. Moreover, the consistency of the deposits which results in numerous artifactual tears during both thick and thin sectioning is characteristic of calcified tissue. There is little doubt that the intracellular and extracellular deposits contain calcium salts, in view of their staining reactions, their hardness, and the presence of crystals.

The earliest changes observed are an increase in the number and size of vesicles and vacuoles in the cells of the proximal convoluted tubules. These vesicular structures appear to originate as invaginations of the luminal surface membranes and suggest increased pinocytotic activity in the proximal tubular cells. Similar vacuoles are also found within the middle and basal portions of the proximal tubular cells. The contents of the vacuoles are initially of low electron opacity. After 1 week, some of these vacuoles contain amorphous deposits of very electron-opaque material which is considered to be of mineral nature while others contain aggregates of crystals whose dimensions are consistent with those of hydroxyapatite (26–30). The larger, basally oriented vacuoles in general contain more mineral than the smaller apically situated vesicles. The failure to obtain adequate selected area electron diffraction patterns for unequivocal chemical characterization of the intracytoplasmic mineral is probably due to the relatively small volume of mineral in any one area. There is no evidence that the mitochondria are directly involved in the formation of the mineral-containing vacuoles.

Extracellular mineral deposits, composed for the

most part of approximately cuboidal 50 Å particles, are present, during the 2nd week of the experiment, near the basal regions of the proximal convoluted tubules. These deposits are first seen in the extracellular phase of the basal infoldings and frequently exhibit a layered arrangement. With growth, these aggregates tend to occupy one of two positions. In the first instance, they may extend within the greatly expanded extracellular phase of the basal infoldings towards the lumen of the tubules. Such growth may be related to the pathogenesis of renal calculi which occur with prolonged hypercalciuria. In the other case, these extracellular aggregates may extend along the basal portion of tubules within the plane of the basement membrane and undoubtedly correspond to the calcification seen with light microscopy in this area. In addition to the small 50 Å mineral particles, occasional elongated crystals similar to those observed in the cytoplasmic vacuoles, are present in the extracellular foci of calcification.

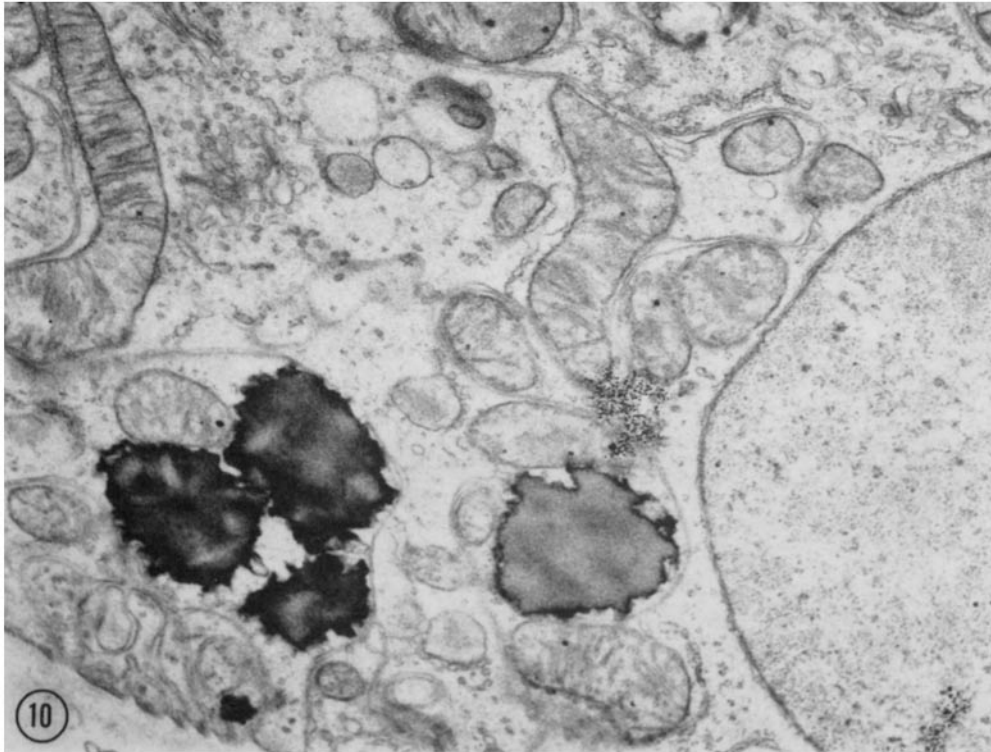
The fact that calcium deposits occur in proximal tubules in these experiments is in agreement with the evidence that the proximal convoluted tubule is the site of calcium reabsorption (31) and is probably directly related to the hypercalciuria associated with hypervitaminosis D. The transport of calcium by the tubules is an active process (31). The ultrastructural correlates of this process are undefined. The fine structural alterations observed suggest several mechanisms for calcium reabsorption by the proximal convoluted tubules. Calcium and perhaps phosphate may be reabsorbed from the lumina of the proximal convoluted tubules and transported within cytoplasmic vacuoles by a pinocytotic mechanism. As an alternate process, calcium may pass across the cell surface membrane directly into the cytoplasm and subsequently accumulate within intracellular vacuoles. This latter mechanism is somewhat analogous to that demonstrated for the sarcoplasmic reticulum of striated muscle. It has been shown that cell fractions consisting of the vesicular and tubular components of the sarcoplasmic reticulum have the ability to actively bind calcium in the presence of adenosino-

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FIGURE 9 A higher magnification electron micrograph of Fig. 8 demonstrates the size and shape of some of the crystals within the vacuole.  $\times 115,000$ .

FIGURE 10 Several subnuclear lipid droplets are seen in the basal aspect of this tubular cell.  $\times 16,000$ .





triphosphate (32-34). It is of interest in this regard that ATPase activity has been demonstrated in pinocytotic vesicles of capillary endothelium (35). Furthermore, the membranes of the cells of the proximal convoluted tubules possess ATPase activity (36). This activity is greater in aminonucleoside-induced nephrosis, a condition in which increased pinocytotic activity has been described in the process of hyaline droplet formation (37).

Both of the above mechanisms are consistent with the morphological findings and with the energetics of calcium reabsorption. It is also possible that these two processes occur simultaneously. The larger basal vacuoles may result from coalescence of smaller mineral-containing vesicles (38).

The interstitial mineral deposits undoubtedly reflect a further sequence in the transport of calcium across the proximal convoluted tubule. The calcium which is absorbed by the cells may subsequently be released into the extracellular space of the basal infoldings. Large mineral-containing intracytoplasmic vacuoles are frequently seen in close apposition to the basal infoldings.

It is well known that a variety of molecular species are reabsorbed in the proximal convoluted tubule by pinocytosis (37, 39, 40). Moreover, it has been shown that hyaline droplets, composed presumably of proteins and amino acids, gain access to the interstitial space at the base of the tubular cells (37, 39). The observations in the present study regarding the uptake of calcium and its subsequent probable transport to the extracellular space are in agreement with these findings.

The dimensions of the crystals within the cytoplasmic vacuoles, as previously noted, are consistent with those of hydroxyapatite. Previous diffraction studies have demonstrated hydroxyapatite in certain forms of pathological calcification (41). With the exception of otoliths (42) and some types of pathological calcification (17, 43-45), normal as well as abnormal calcification in vertebrates generally implies the presence of hydroxyapatite.

The deposition of calcium within the cells of the proximal convoluted tubules occurs in the absence of collagen fibers, which were noted by Glimcher

*et al.* (29) and Neuman and Neuman (46) to serve as the nucleating centers for crystal formation in the presence of metastable solutions of calcium and phosphate. More recently, Glimcher and Krane have emphasized the importance of phosphate groups *per se* in the formation of hydroxyapatite crystals (47, 48). The acid phosphatase present in renal tubular reabsorption vacuoles or lysosomes (49, 50) may provide free phosphate groups for hydroxyapatite crystallization. The previously discussed ATPase of proximal tubular cells may play a similar role in this regard (36).

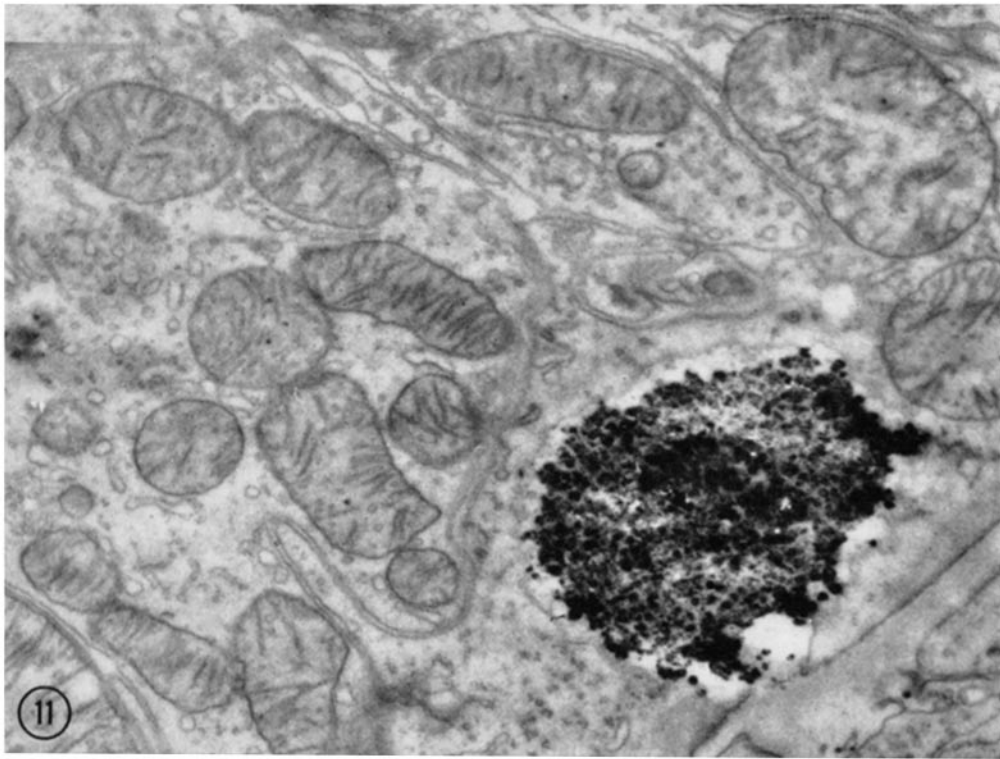
The crystals forming the extracellular mineral deposits appear to be generally cuboidal in shape and measure approximately 50 Å. The extracellular crystals occasionally exhibit the elongated profiles of both hydroxyapatite in mature bone (26-28) and the proximal tubular intracytoplasmic aggregates described in this study. The dimensions of the more frequent, small extracellular crystals are consistent with those of hydroxyapatite found in the early phases of *in vivo* and *in vitro* collagen mineralization (29, 51). Selected area electron diffraction patterns obtained from the interstitial calcific deposits disclose six diffraction maxima. Dr. Aaron S. Posner, who examined these diffraction patterns, indicated that they are oriented patterns of some crystalline substance which cannot be definitely identified from the available data. The absence of major calcite diffraction maxima excludes the presence of this compound in these deposits. All the reflections, except 6.2 and 3.1 Å, can be indexed as apatite. However, a strong apatite reflection of 3.44 Å is missing. Therefore, one cannot say from the present data that this material is apatite, although a mixture of apatite and another phase (related to 6.2 and 3.1 Å maxima), though unlikely, is not impossible. This latter possibility received some support from the positive von Kossa staining and the absence of carbonates in the calcific deposits. In view of the small dimension of the extracellular crystals, it is also possible that the diffraction patterns are derived from other salts rather than these crystals.

The difference between the usual crystalline

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FIGURE 11 An extracellular mineral deposit is noted in the basal infolding of this cell.  $\times 18,000$ .

FIGURE 12 Similar to Fig. 11. The mineral particles form a layer at the periphery of the deposit.  $\times 24,000$ .



habit of the extracellular aggregates in the basal infoldings and that of the elongated crystals within the cytoplasmic vacuoles may be due to different rates of crystal growth at the two sites. It is well known that the growth rate and therefore size and shape of synthetic hydroxyapatite crystals is dependent in part on pH and other physicochemical factors (52). It is possible that a pH gradient exists between the intracytoplasmic vacuoles and the interstitial fluid at the base of these acid-secreting cells of the proximal convoluted tubules (53). Such differences in the physicochemical environment might account for the observed variation in crystal shape.

Sobel *et al.* have suggested that mucopolysaccharides can catalytically induce the aggregation of calcium and phosphate ions to form the initial crystals of hydroxyapatite (54). Massive doses of vitamin D result in the mobilization of mucoprotein as well as mineral from bone (33). The presence of mucopolysaccharides is suggested by PAS-positive material within the calcific deposits in thick sections of the Araldite-embedded material.

It should be emphasized that, aside, from some flattening or compression of the cells with dilatation of the proximal tubular lumina, there is no evidence of cell injury. It is our impression that the changes observed appear to be an exaggeration of normal transport phenomena and are somewhat analogous to those seen in experimental and human proteinuria where there is hyaline droplet formation. In these latter instances, it has been pointed out that the hyaline droplets are related to increased protein reabsorption by the tubular cells, rather than to degenerative cellular alterations (39, 55).

Our observations differ somewhat from those described by Engfeldt *et al.* in a study of metastatic calcification within the kidney produced by the administration of parathormone (56). The early changes consisted of increased numbers of micro-

bodies in the proximal tubular cells with concomitant reduction in numbers of mitochondria. Subsequently, those authors described the formation of intracellular hyaline bodies which enlarged, burst into the lumen of the tubules, and formed hyaline casts which served as centers for the precipitation of apatite crystals during the formation of renal calculi. The disagreement between this latter study and the present one may be related to differences in the agents used, in the experimental models, the duration of the experiments, and the techniques employed.

The deposition of calcium in other parts of the nephron following a more prolonged period of hypervitaminosis D described by others (1-13) may be explained by the fact that the long term deposition of calcium within the proximal convoluted tubules so injures these tubules that calcium is no longer reabsorbed at this site. The calcium may then be concentrated in the more distal portions of the nephron as water is reabsorbed until the solubility products of calcium-phosphate salts are exceeded, resulting in salt precipitation (13).

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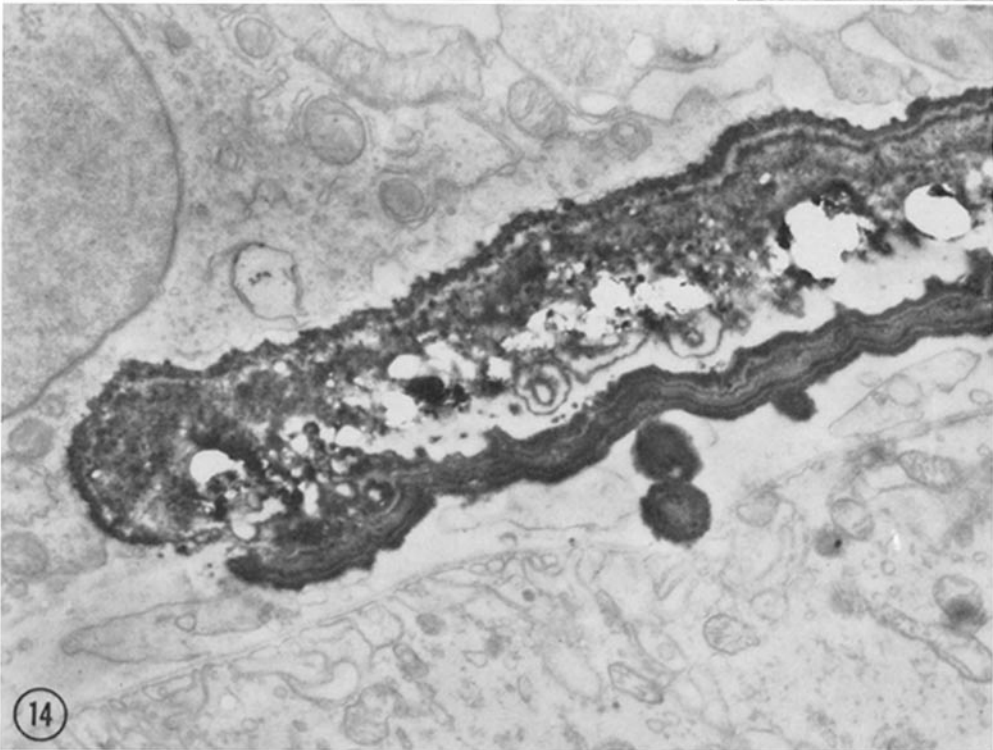
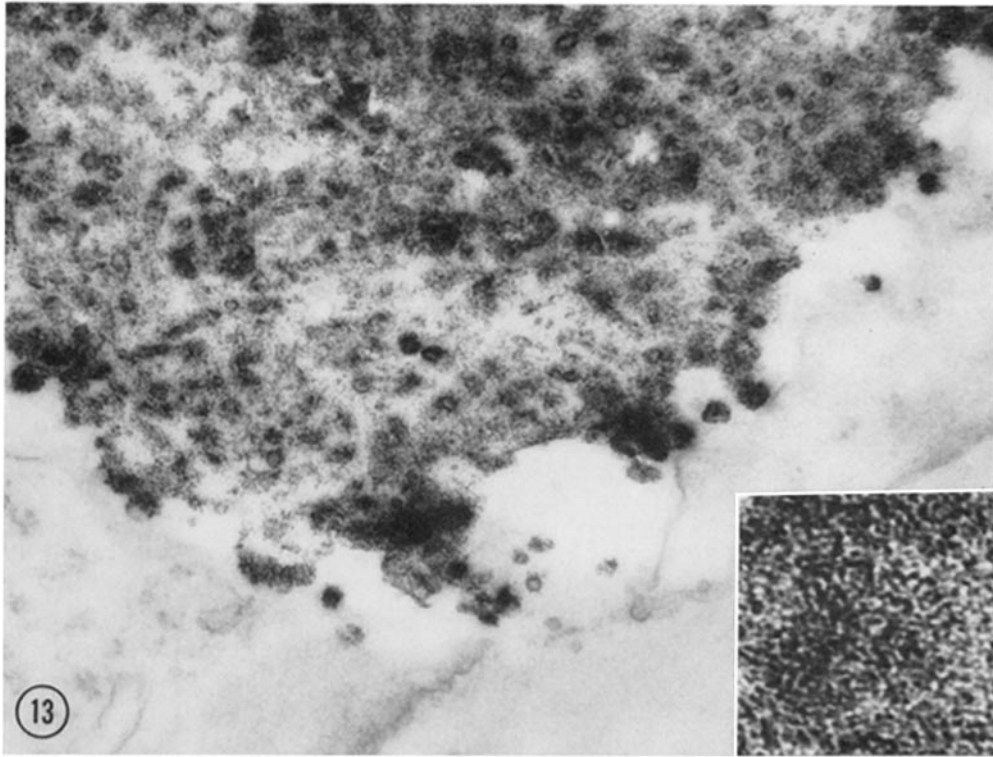
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FIGURE 13 Higher magnification electron micrographs of Fig. 11 reveal the usual size and shape of the electron-opaque crystals.  $\times 48,000$ ; Inset,  $\times 138,000$ .

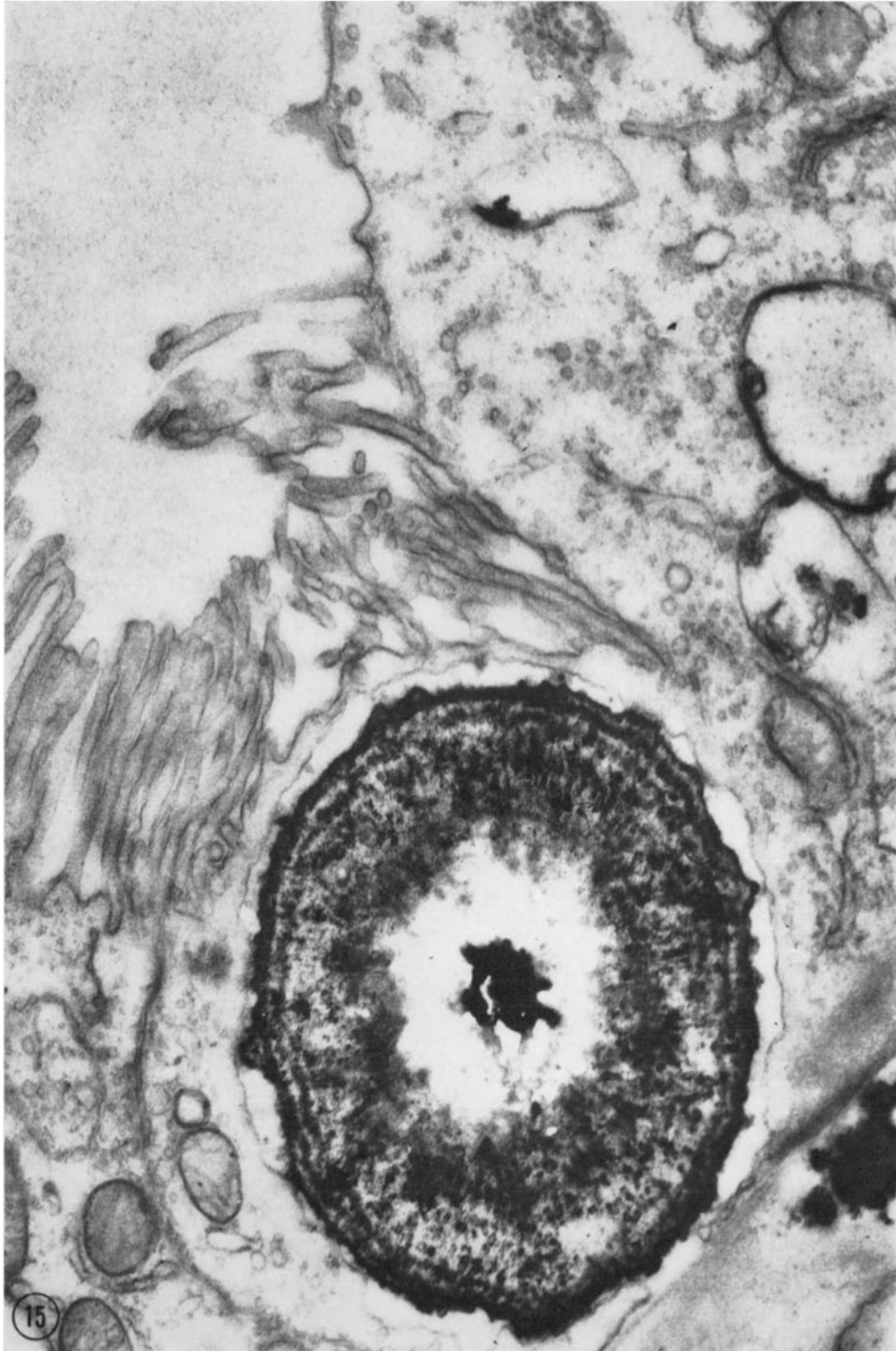
FIGURE 14 A large extracellular mass of mineral displays a laminated arrangement and extends for a considerable distance along the plane of the basement membrane.  $\times 12,000$ .



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FIGURE 15 A lamellated calcific mass in a greatly expanded basal infolding extends from the basement membrane to the luminal portion of the cell. Several cytoplasmic vacuoles containing electron-opaque material are present. A small mineral deposit is seen in the region of the basement membrane at the lower right.  $\times 25,000$ .

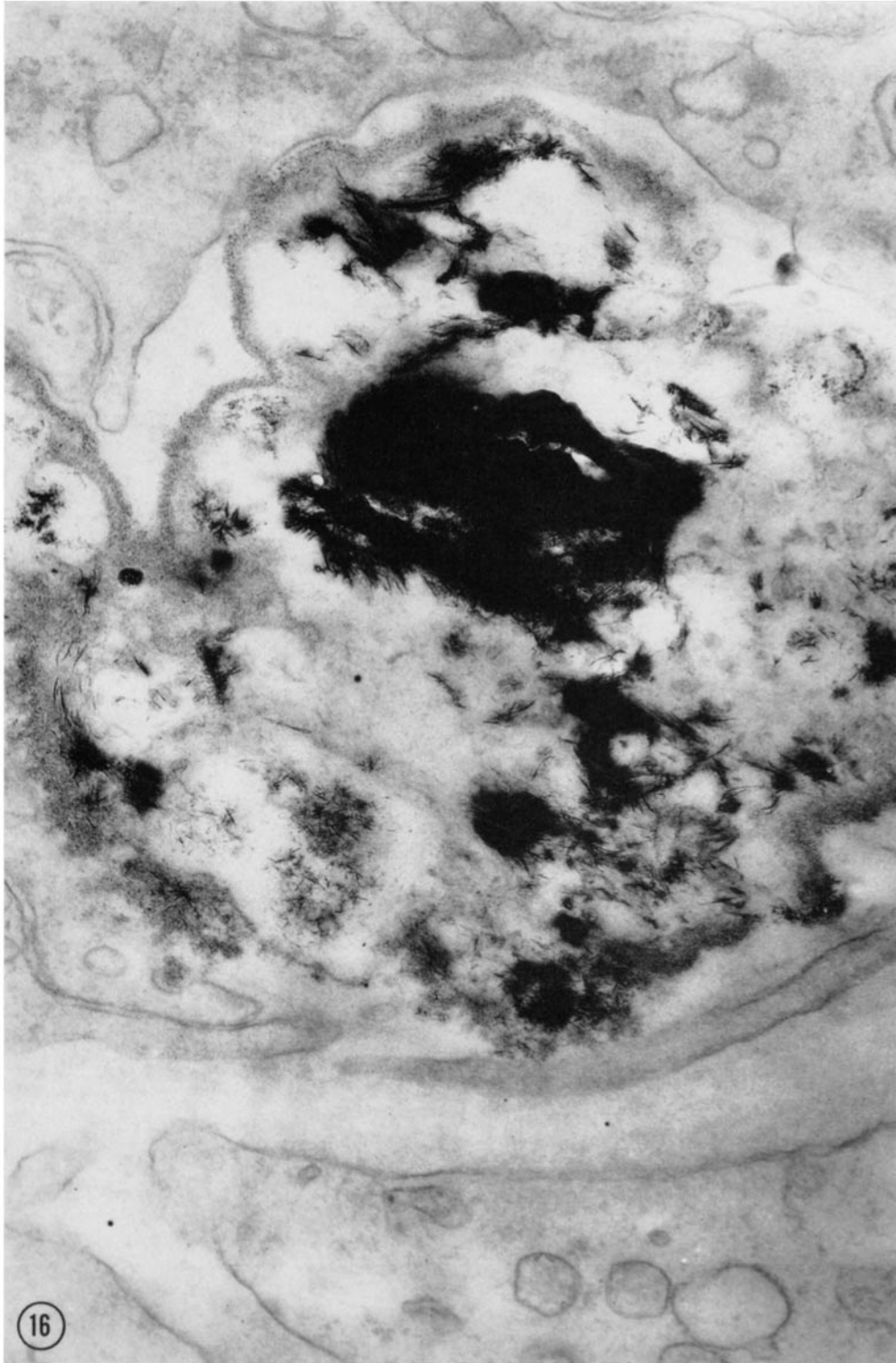


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FIGURE 16 This electron micrograph shows an extracellular mineral deposit that is made up of elongated as well as smaller crystalline particles.  $\times 49,000$ .





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