LIPOPROTEIN GRANULES IN THE CORTICAL

COLLECTING TUBULES OF MOUSE KIDNEY

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ABSTRACT

The light and, to a lesser extent, the dark cells of the cortical collecting tubules in mouse kidney contain a great number of granules which according to histochemical tests are composed of phospholipids and proteins. These granules are bounded by a triple-layered membrane measuring approximately 75 A across, and contain one or several crystals with a hexagonal or square lattice. These crystals are built up of rod-shaped units, which appear dense after osmium fixation, measure about 48 A in diameter, and are separated by a light interspace of similar dimensions. The mean center-to-center distance of the rods is about 96 A. The structure is explained as a lipoprotein crystallized within a membrane-bounded vacuole. No relationship between these granules and mitochondria was found. The physiological significance of the granules remains unknown.

INTRODUCTION

Lipid granules have been described in many parts of the nephron in laboratory animals (1–4). Fischer (5) and Segawa (6) found lipid granules regularly in the cells of the collecting tubules of man, and both authors noted that only a few cells contained them and that they were always separated by cells free of fatty material. In mice, Bachmann (4) found no fat at all, and Fischer (5) mentioned only traces of it. Sjöstrand (7) described small autofluorescent granules massed around the nuclei of some cells of the proximal collecting tubule, and others that stained intensely with iron hematoxylin. He noted that cells containing an abundance of granules appeared dark and thought that they corresponded to the dark cells first described by Schachowa (8). Sjöstrand (7) assumed that these granules were of "plastosomal" origin because they stained as intensely with iron hematoxylin as mitochondria. Oliver (9) also ascribed the dark color of the intercalated or dark cells to the presence of coarse metachromatic granules.

With the electron microscope the proximal or cortical portion of the collecting tubule (portio reuniens) has been investigated in the rat (10), rabbit (11) and mouse (12, 13). The fine structure of the light and dark cells of this part of the nephron has been reviewed by Rhodin (14). None of these authors commented on certain characteristic granules occurring regularly in light and dark cells in mice, with the exception of Clark (13) who described dense cytoplasmic bodies the size of mitochondria in some cells of the collecting tubule and suggested that they were mitochondria "altered by the accumulation of a dense or osmiophilic material."

In this paper we will describe the fine structure of these granules and present some histochemical data on their composition.

MATERIALS AND METHODS

Material for these observations was collected from 3 normal adult female white Swiss mice of The Rockefeller Institute stock and of 19 mice which had received intraperitoneal injections of ox hemoglobin.

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The tissues were prepared for electron microscopy with the methods detailed in a previous paper (15); block areas containing collecting tubules were selected under a microscope with reflected light illumination. The rest of these kidneys, and the kidneys of 6 other male and female white Swiss mice of different stocks were fixed in various fixatives given in Table I.

A Siemens Elmiskop I operated at 80 KV with 50 μ molybdenum objective apertures and single or double condensor was used for electron microscopy. The study is based on the examination of 295 plates taken at magnifications between 2400 and 150,000. The instrumental magnification was calibrated with a grating replica.

OBSERVATIONS

The cortical collecting tubules are characterized by a wide lumen and a ligning of cuboidal or pyramidal cells. The basal part of the cells is broader and rests on a basement membrane; the apical part is narrower and bulges sometimes into the lumen (Figs. 1 and 2). The cell membrane is triple-layered (Fig. 8) and of unit membrane (16)

dimensions measuring between 70 and 80 A. Light (Figs 5 and 6) and dark (Figs. 5 and 6) cells were found in agreement with the observations of Clark (13) and Rhodin (14).

The granules now to be described have been found mainly in the light cells, but they do occur also in the dark cells. They are present in almost every collecting duct examined, but their distribution varies considerably. Some cells contain no granules, some only a few, whereas some are loaded with them. In the latter case the granules occupy mainly the apical half of the cell and only seldom occur in the basal half. The profiles of these granules are round, oval, or angular (Figs. 6 and 7), but very irregular forms were also seen. Their diameters lie between 0.5 and 3 μ; the most frequent value is around 1 μ. The granules are easily recognized in 0.5 μ thick methacrylate-embedded sections in the phase contrast microscope (Fig. 1). In fresh frozen sections they are colorless, have a high refractive index, and are birefringent. In paraffin sections they are best seen with the PAS procedure because of their intense red staining (Fig. 2).

Explanation of Figures

Figs. 1 to 4 are light micrographs, the rest are electron micrographs from osmium-fixed, methacrylate-embedded sections covered with a thin layer of carbon (40). Fig. 5 is from an unstained section, Fig. 6 is from a section stained with 2 per cent uranyl acetate (39), and the rest from sections stained with lead hydroxide (37, 38).

FIGURE 1

Phase contrast micrograph of a collecting duct. Dark granules lie in the apical parts of several cells lining the tubule, and some are indicated by arrows. Methacrylate-embedded section from osmium fixed material. × 950.

FIGURE 2

PAS-reaction in collecting duct. Granules in the apical parts of the cells lining the tubule stain intensely red. Arrows indicate supranuclear location of granules in two cells. Paraffin-embedded section from material fixed in neutral formalin. × 1200.

FIGURE 3

Baker reaction in collecting duct. Two cells lining the tubule contain granules which stain black (arrows). Frozen section from material fixed in neutral formol-calcium. × 800.

FIGURE 4

Luxol fast blue stain in collecting duct. Four cells lining the tubule contain granules which stain blue (arrows). Frozen section from material fixed in neutral formalin. × 800.
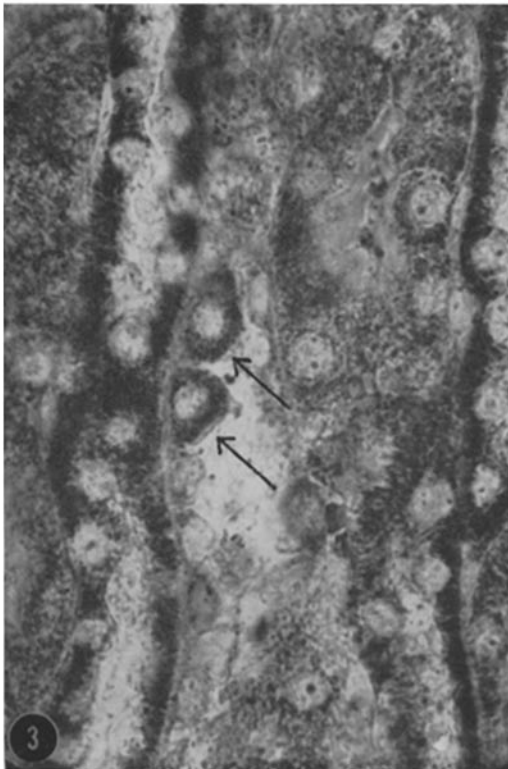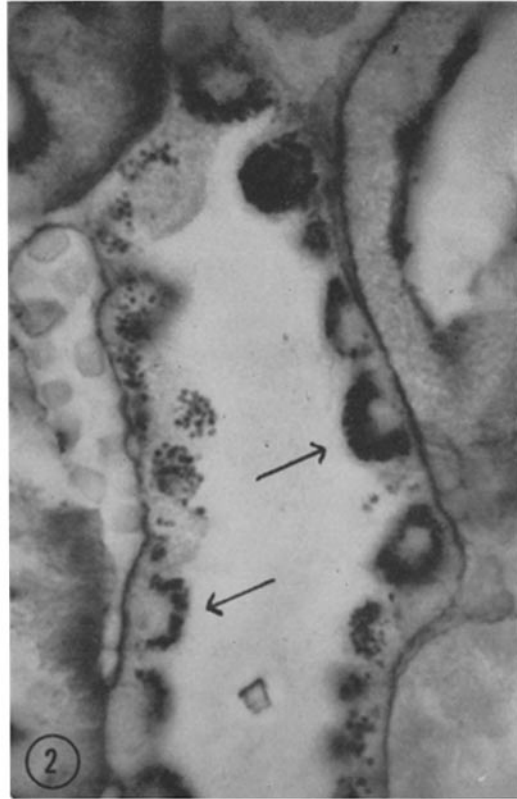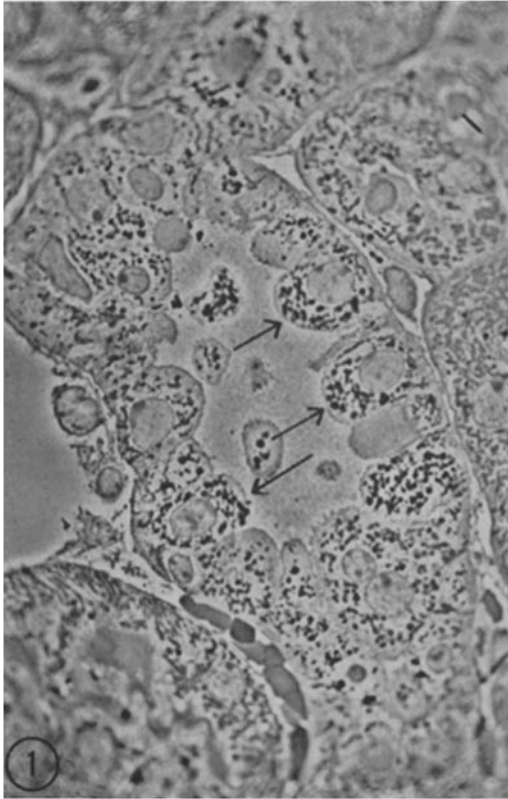
The histochemical tests carried out on these granules and the results obtained are summarized in Table I.

The results of the tests given in Table I demonstrate that the granules contain acidic (test A 2), unsaturated (test B 1), lipids (tests A 1, 3; B 2, 3), primarily phospholipids (tests A 2, 4), and also some protein (tests B 4–8).

Electron microscopy reveals a highly ordered fine structure comparable to a crystalline lattice. The granules are surrounded by a triple-layered membrane with two dense lines and a middle light band, measuring 75 A across (Figs. 7 and 9). In the interior, separated from the membrane by a light interspace *ca.* 200 A wide, several patterns were observed, namely:

1. Dense roundish dots packed in a slightly distorted hexagonal array (Figs. 7, 10, 11, and 14) and separated at all sides from each other by a less dense interspace. Less frequently, an almost quadratic array was found in other granules (Fig. 15).

2. Straight or slightly curved parallel alternating dense and less dense bands which frequently transverse the entire granule (Figs. 7, 9 to 11).

3. Regions in which alternating dense and less dense bands are indistinct and which blend into regions appearing homogeneous (Figs. 7, 9, and 11).

4. Regions with alternating dense and less dense bands arranged in a few parallel pairs or in irregular form resembling whorls or finger-print patterns (Figs. 7 and 10).

The mean of the center-to-center distance between the dots and between the dense bands is 96.21 ± 0.5 A (s = 4.2). This value was obtained from 60 measurements on prints magnified 3 times from plates taken at magnifications between 40,000 and 150,000. Measurements were carried out by counting the number of dots or bands over a known distance and included 6 to 58 periods in one measurement depending on the magnification. Through focus series were obtained at magnifications between 40,000 and 150,000. Measurements on single dense and less dense bands and on dots showed that the width of the dense and less dense bands and of the dots and the light interspaces between them was approximately the same. Thus, each dot, dense band, and light interspace measures approximately 48 A across. The repeating period of the bands in pattern No. 4 is markedly higher than in patterns Nos. 1 and 2, and is approximately 140 A.

Small granules show only one or two of the patterns mentioned, most frequently Nos. 1 and 2. In larger granules many different patterns may be seen, and in this case they are always separated from each other by a light interspace measuring between 200 and 1000 A (Figs 7 and 11). In one large granule 12 separate regions were observed; four of these showed dots, three light and dense bands, and five had no distinct structure.

The patterns observed suggest the presence of a single or of several (up to 12) crystals or imperfect crystals lying at different angles within a membrane-bounded vacuole. The structure of these crystals cannot be determined with certainty from their aspect in sections alone. One possible assumption is that each crystal is composed of (cylindrical ?) rods arranged parallel to their long axis in a hexagonal or, more rarely, in a square array. If such rods are sectioned normal to their long axis, a dot pattern (No. 1) will result. A slight tilt of the rods with respect to the plane of the section and the direction of the electron beam will result in the appearance of bands. A simple geometrical

FIGURE 5

Low power micrograph of cortical collecting tubule which is lined by cuboidal or pyramidal cells. Dark cell (*d*) contains many mitochondria in the apical region, light cell (*l*) has no mitochondria above the nucleus. Very dense granules are visible in some light cells and in the dark cell. × 3800.

FIGURE 6

Oblique section through cells lining a collecting tubule. Very dense, oval, or angular granules (*g*) are seen in the cytoplasm mainly of the light cells, and a few also in the dark cell (*d*). Mitochondria (at lower left and in dark cell) are much less dense than the granules. × 17,000.
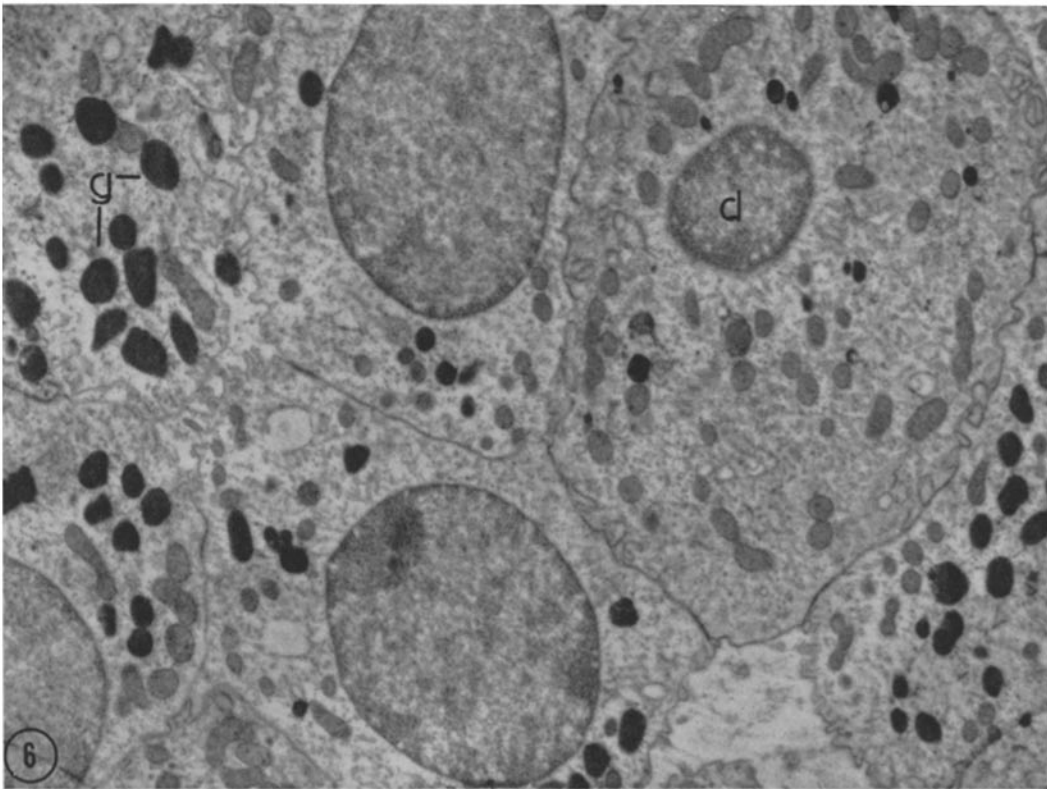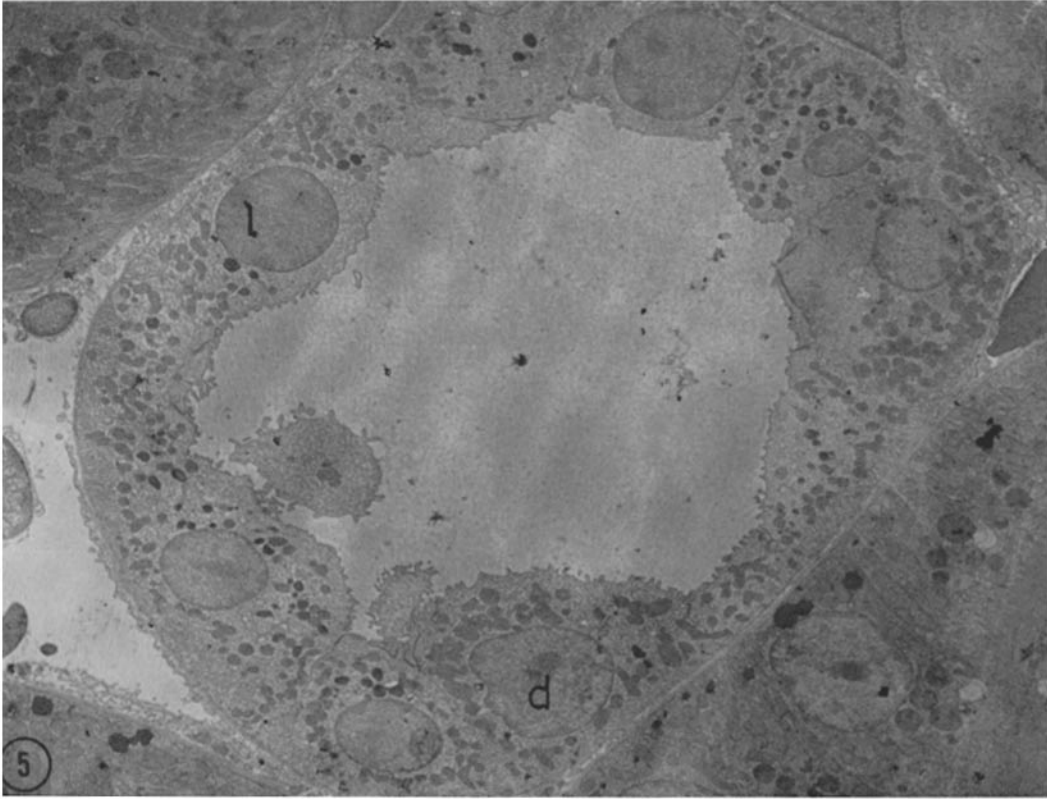
approximation shows that in a section 500 A thick two rods of a diameter of 50 A and spaced at a distance of 50 A from each other will appear as band 260 A long under an angle of tilt of 80° with respect to the plane of the section; at an angle of tilt of 50° the two rods will appear as band 580 A long. Since most of the gray and the silver sections are thicker than 500 A (28) and since many rods are in alignment as seen from the aspect of the dot patterns, this explanation of the band patterns is very plausible. Stereo pictures taken at a magnification of 74,000 under a stereo angle of 10° show a dot pattern in one picture which seems to change into a band pattern in the other (Figs. 12 and 13). The result of the stereo micrographs is, however, less convincing than one would have expected, and this remains unexplained for the moment.

A merging of a dot into a band pattern, in the direction of the long axis of the bands, should result if the rods were either curved or not perfectly parallel in the plane of their long axis but had slightly increasing tilt so that the section passes from a direction normal to the long axis of the rods to one intersecting them obliquely. Such a change from dots to bands can be seen in Figs. 10, 14, and 15.

The band pattern should also be seen more frequently than the dot pattern because of the greater probability of oblique sectioning, and this is, indeed, the case. Regions with indistinct bands or homogeneous regions (No. 3) will result from very oblique cutting through parts where the rods are not perfectly aligned. No halfing of the main period can be expected by cutting at different planes through the lattice because the diameters of dense dots or bands and light interspaces are approximately the same.

A second possibility would be that the crystals are composed of (spherical ?) subunits with a diameter of *ca.* 48 A lined up like beads on a string and spaced at a distance of *ca.* 48 A. This question could be settled definitely in sections not

thicker than 50 A which were not obtained. In thicker sections one should observe, however, at the periphery of an obliquely cut lattice, a change from a dot pattern to a band pattern in the direction of the long band axis. Such a pattern can be clearly seen in ferritin crystals composed of individual particles of ferritin in several figures (Nos. 11/1, 12/2, 14/3) of the paper of Favard and Carasso (29). Here, a dot pattern at one edge of the ferritin crystal changes into a band pattern in the interior and into a dot pattern again at the opposite edge. In our smaller granules containing only one crystal and cut obliquely through the lattice as indicated by the appearance of band patterns, the bands run across the entire diameter of the granule, and a change from dots near the limiting membrane through bands to dots at the opposite edge was not observed. It seems more likely, therefore, that the crystals are built of rods than of aligned subunits.

It cannot be determined whether all the rods have the same length. Bands running across the entire diameter of the granule can as well result if individual rods are aligned behind each other but shifted in the direction of their long axis. The whorl-like bands with a higher repeating period (No. 4) were rarely seen. Only one granule among the many hundred examined displayed exclusively such a structure. A dot pattern was never observed in these thicker bands and we assume that they consist of membranes of a different kind. Their relation to the crystalline structures remains unclear; they are always separated from them by a light interspace and do not seem to merge with them.

A relationship between the granules and mitochondria can be ruled out. No clue was obtained as to their origin or development.

DISCUSSION

The dark cells contain mitochondria in their apical region and the intense staining with iron

FIGURE 7

Several dense granules of varying shape in a light cell. Each granule is bounded by a membrane. The large granule at left shows dot and band pattern in its upper part, a band pattern of lesser contrast in its lower middle part and a band pattern near lower end. Note two light interspaces running across the granule in the lower half. The two granules at lower left show very regular band patterns. Nucleus of cell is at left and a mitochondrion at lower right. X 65,000.
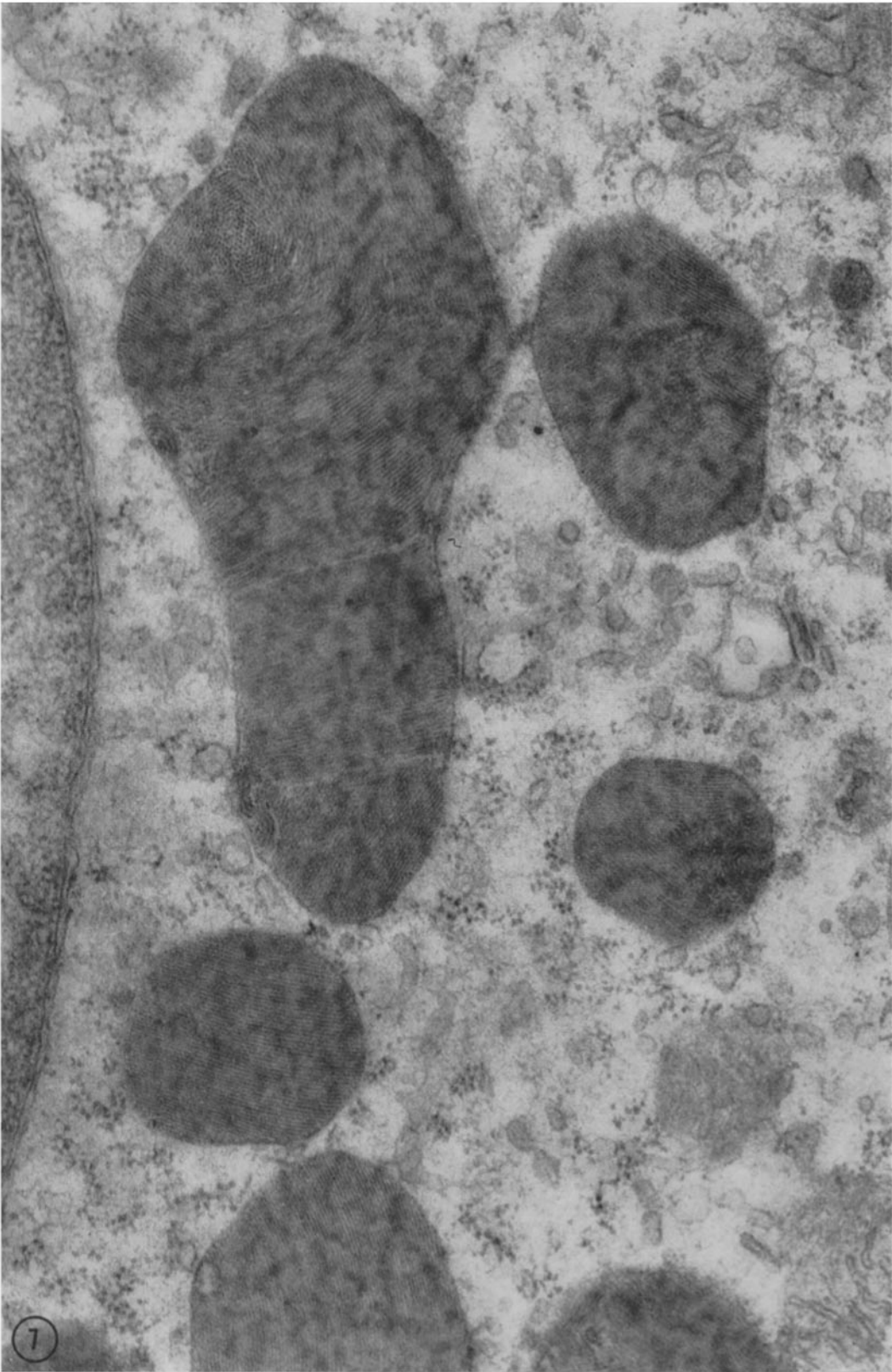
hematoxylin depicted by Sjöstrand (7) and Rhodin (14) could, indeed, indicate the presence of mitochondria. This is confirmed by a high activity for certain dehydrogenases, mainly succinic dehydrogenase and DPN-diaphorase in dark cells (30). With iron hematoxylin mitochondria and crystalline granules cannot be distinguished. Sjöstrand's (7) Figs. 62 and 70 and Oliver's (9) Figs. 8 and 32 correspond exactly to our Figs. 1 to 4. It is misleading, therefore, to classify all cells that contain granules as dark cells unless the mitochondrial nature of the "granules" is proven.

The membrane surrounding the granules is triple-layered and has the dimensions of a unit membrane (16). Whether this membrane is a pinched-off derivative of the cell membrane which has the same dimensions in our material, cannot be decided because continuity was not observed. The presence of one or several crystals within a membrane-bound vacuole indicates that crystallization is brought about by peculiar conditions prevalent inside the vacuole which favor concentration and purification. The occurrence of a crystalline lattice in a predominantly phospholipid material, which is known to form smectic crystals, has not been reported previously.

The following deliberations about the molecular architecture of the crystals have only the value of a working hypothesis. The histochemical data permit the statement that the granules contain little protein and a large amount of unsaturated phospholipids. It cannot be decided whether a true lipoprotein with salt-like linkages between the acidic polar groups of the phospholipids and

basic amino groups is present or whether the protein is only adsorbed onto the polar groups of the phospholipids.

Recent work on myelin figures in globin solutions (31) and on the osmium distribution in myelin figures (32) would permit the assumption that the dense rods measuring 48 A contain in their core chains of proteins arranged parallel to their long axis and at their periphery the polar groups of radially disposed phospholipid molecules. Stoeckenius (31) found the outer heavy dense line produced by the addition of globin to phospholipids 25 to 50 A thick. The latter value corresponds to the thickness of the dense dots or bands. Finean (33) has obtained a value of about 9 A for the end group of cephalin. This would leave about 30 A for the protein in the rods. The light interspace measuring about 48 A could correspond to the hydrophobic side chains of a bimolecular leaflet of phospholipids arranged in planes normal to the long axis of the rods and sticking out radially towards each other. The width of the light interspace is, however, twice as large as the one found by Stoeckenius (31) in phospholipid myelin figures. Nothing is known about the chain length and the degree of folding of the fatty acid chains in our material. To fit our assumptions we would have to postulate either the presence of long chained lipids such as sphingomyelin or cerebrosides, or a considerable unfolding of shorter chained lipids. Palmer, Schmitt, and Chargaff (34), by x-ray diffraction on dry material, found a spacing of 59 A for a cephalin-histone complex, and assumed that a monolayer of pro-

FIGURE 8

Microvilli of a light cell in collecting tubule the lumen of which is at right. A triple-layered cell membrane is visible over parts of the microvilli. × 109,000.

FIGURE 9

Part of a dense granule in a light cell with a pattern of curved bands at lower right merging into a band pattern with less contrast in the middle part. A triple-layered membrane of the same dimensions as the cell membrane is visible in parts around the granule. × 190,000.

FIGURE 10

Part of a dense granule in a light cell. Between and in the directions of the arrows, the hexagonal dot pattern at right merges into a band pattern at left. Such a change will result when aligned rods are cut under an increasing angle of tilt. The light interspaces have approximately the same width as the dense dots and bands. Near the right margin a region with broader dense and light bands is visible. × 450,000.
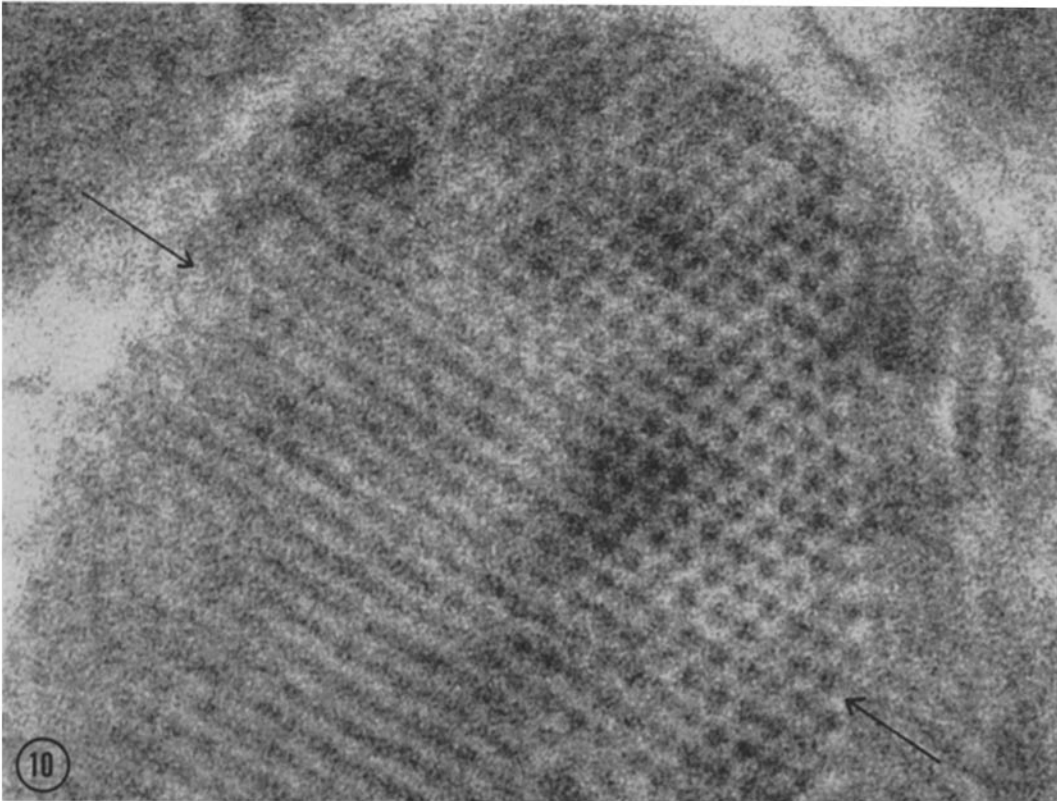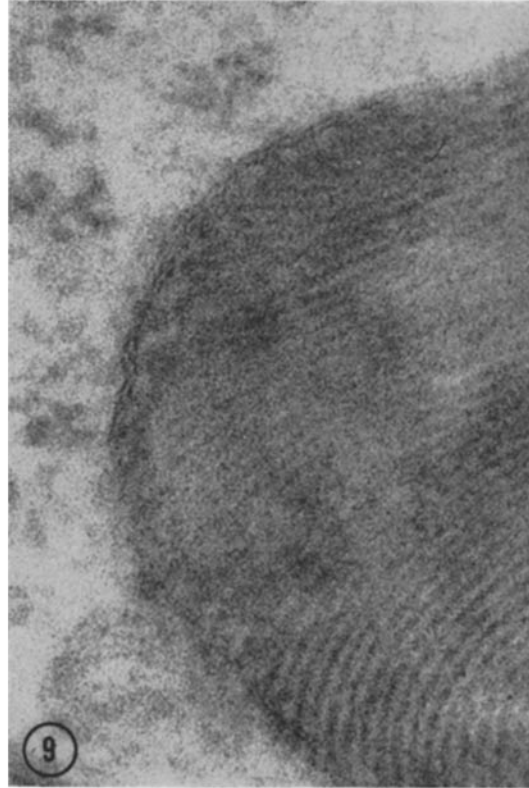
TABLE I

A. Tests conducted on frozen sections of kidney fixed in 1 per cent calcium acetate in 10 per cent formalin, or in phosphate-buffered (pH 7.0) formalin

	Staining of granules
1. (a) Sudan black B in propylene glycol (17)	Blue-black
(b) Sudan black B in 60 per cent triethyl phosphate (18)	Blue-black
(c) Sudan black B, method for masked lipids (19)	Blue-black
Controls: Extraction with absolute pyridine at 60°, 18 to 24 hrs.	Negative
Extraction with acetone, 60°, 1 hr.	Weakly blue
2. Nile blue sulfate (20, 21)	Blue
Controls: Extraction with acetone, 60°, 1 hr.	Weakly blue
3. Copper phthalocyanin method (luxol fast blue) for myelin (22)	Blue (Fig. 4)
4. Acid hematein test (Baker) (23)	Black (Fig. 3)
Controls: Extraction in pyridine, 60°, 24 hr.	Negative

B. Tests conducted on paraffin sections of kidney fixed in buffered 10 per cent formalin (pH 7.0) or in Carnoy's solution at 0-°4°

	Staining of granules
1. PAS reaction (23)	Red
Controls: (a) amylase digestion at pH 6.0	Red
(b) acetylation in absolute acetic anhydride, 26°, 3 hr.	Red
(c) bromination (2.5 per cent bromine in CCl₄)	Negative
2. Sudan black B for bound lipids (modified). Deparaffinized Sections brought to water were rinsed with acetone and stained with Sudan black B in propylene glycol or in 60 per cent triethyl phosphate for 30 to 60 minutes. Tissue fixed in Orth's fluid was also examined.	Blue-black
3. Stain for myelin. Weigert method	Blue-black
4. Acid fast green (0.1 per cent fast green in acetate buffer, pH 2.5)	Green
5. Alkaline fast green (24), modified	Green tinge Very rarely
6. Naphthol yellow S (25)	Yellowish, slightly more intense than in cytoplasm
7. Mercuric bromphenol blue (23)	Blue
8. Millon reaction, modified (26)	Weakly orange
9. Sakaguchi reaction (27)	Inconclusive

tein was sandwiched between the two polar groups of the lipids. This would leave about 41 A for the two fatty acid chains if the value of Finean (33) for the polar groups of cephalin is substituted and an intercalation of the protein monolayer between the polar groups is assumed. The value of 41 A is close to the value of 48 A for the light interspace in our fixed material. Müller (35)

FIGURE 11

Dense granule with a slightly distorted hexagonal lattice near center, assumed to have resulted from cutting normal to the long axis of rods. Peripherally, the dot pattern changes to a band pattern which still shows hexagonal arrangement. Further outwards, several curved band patterns and regions without ordered structure are visible. Arrow indicates direction of compression during sectioning, and also points to the membrane surrounding the granule. X 260,000.

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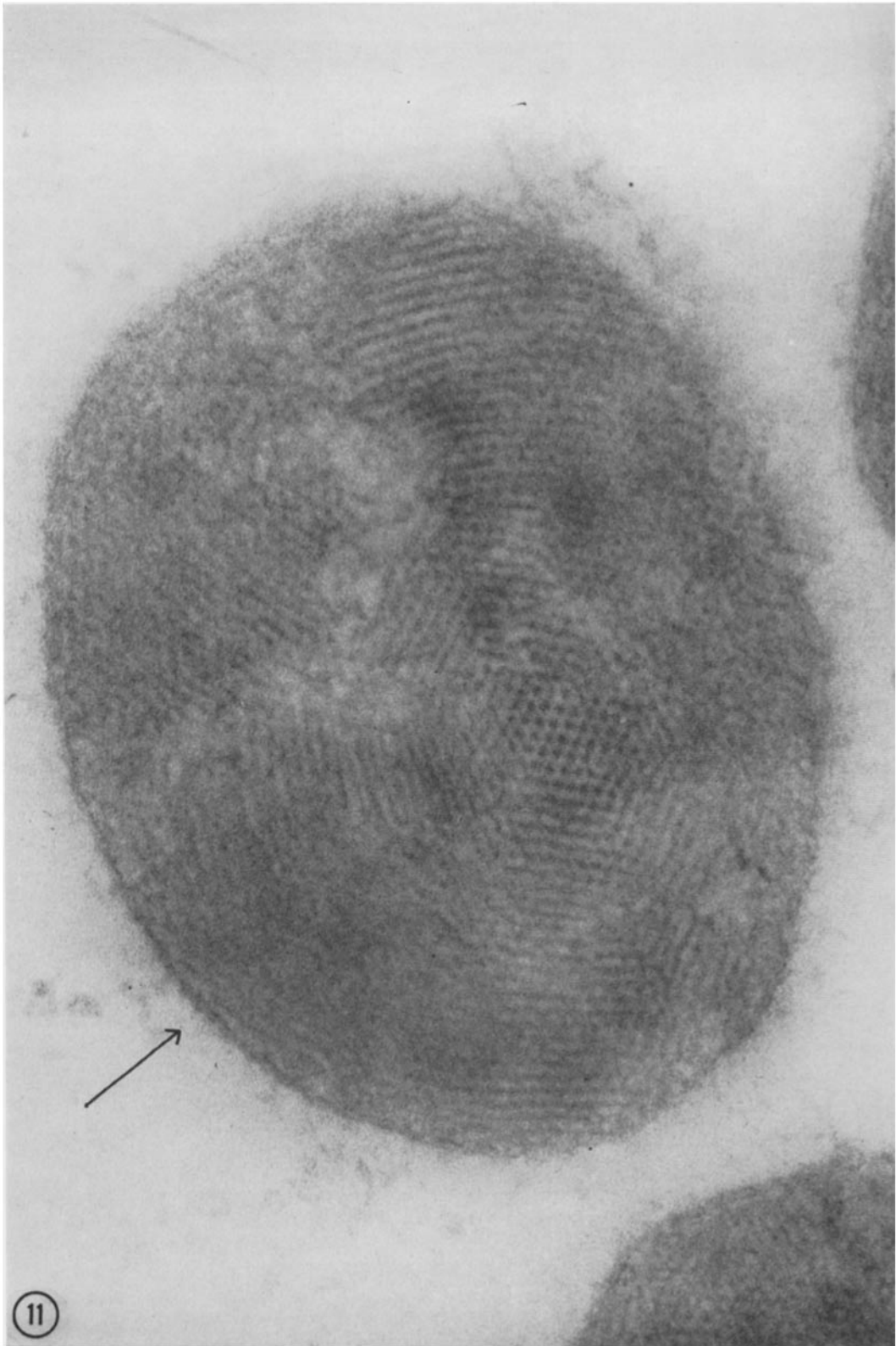
found a value of 48.8 A for the thickness of a double layer of stearic acid in a single crystal. Since our material shows a higher degree of crystalline order than myelin figures which are fluid in tangential direction, two C-18 fatty acid chains could account for the width of the light interspace.

Cellular alterations occur in the collecting tubule during electrolyte imbalance (36) and this part of the nephron cannot be considered merely as a urine collecting and conducting system. The observations reported in this paper would indicate that the cells of the collecting tubule can store lipids and proteins in membrane-bounded vacuoles and purify them by crystallization. Pending clarification of the physiological significance of this finding, it remains surprising that cells belonging to "the neglected tribe of kidney cells" (9) harbor a highly ordered structure which was previously known only as a modest granule or fat droplet.

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Figures 12 and 13

The same region (compare circles) in a dense granule, micrographed under a stereo angle of 10°. The object holder was tilted in the direction indicated by arrows. The dot pattern in Fig. 12 changes into a band pattern in Fig. 13. × 600,000.

Figure 14

A higher power view of the hexagonal dot pattern in the center of the granule shown in Fig. 11. × 485,000.

Figure 15

A square to rhombic dot pattern from another granule. × 485,000.
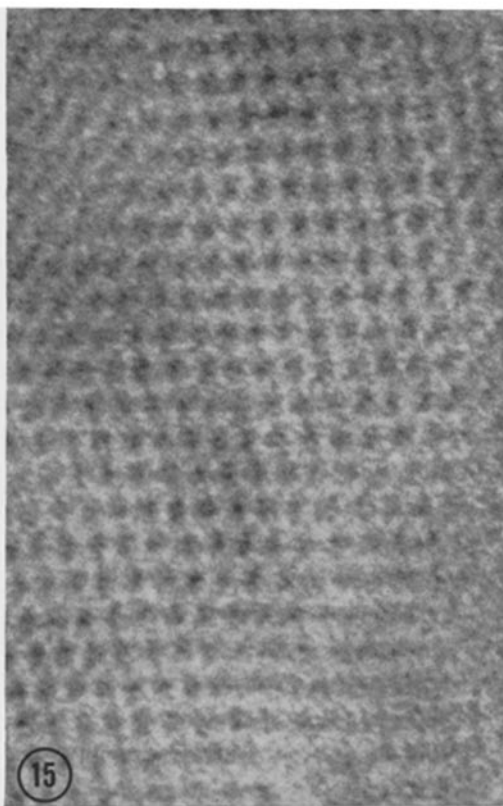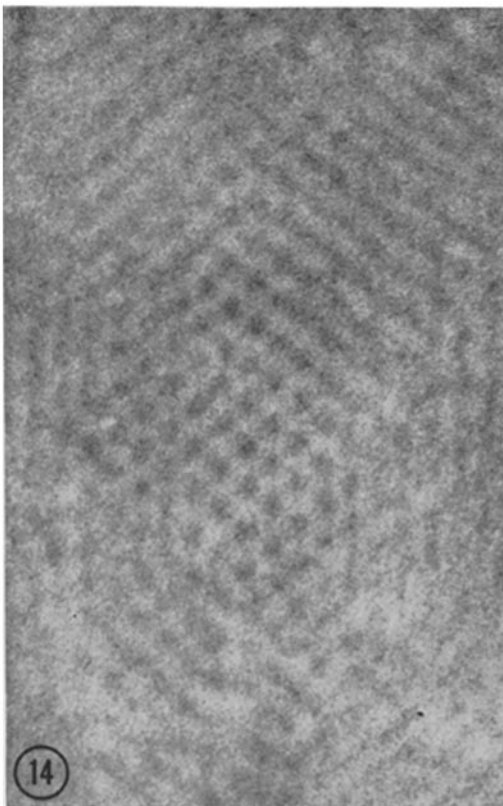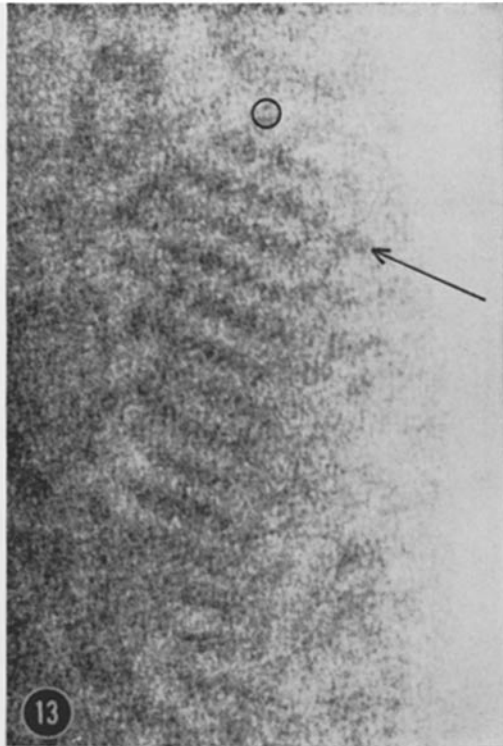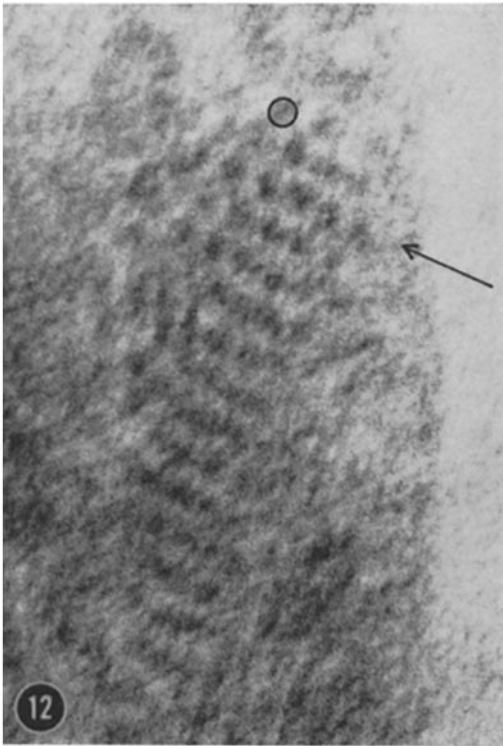
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