CYTOLOGICAL ALTERATIONS RELATED TO STIMULATION OF THE ZONA GLOMERULOSA OF THE ADRENAL GLAND

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ABSTRACT

The structure of the zona glomerulosa of the rat adrenal gland stimulated by sodium restriction has been studied by light and electron microscopy. The major changes observed during the course of the experiment in stimulated glands involve cytoplasmic droplets, mitochondria, and the endoplasmic reticulum. There is a progressive decrease in the number of cytoplasmic droplets of low electron opacity. Numerous, greatly elongated mitochondria containing parallel arrays of tubules are noted. These tubules extend from within the mitochondria through gaps in the mitochondrial-limiting membranes into the cytoplasm. In addition, amorphous intramitochondrial deposits, possibly aldosterone precursors, are seen. Increased amounts of smooth-surfaced endoplasmic reticulum, often showing complex arrangements, are another feature of the stimulated zona glomerulosa. Other alterations include the presence of large numbers of dense bodies as well as cytoplasmic droplets of high electron opacity. These observations are discussed in relation to the biosynthesis of aldosterone.

The fine structure of the normal adrenal cortex has been described in several species (1–5). In addition, the changes occurring in the zona fasciculata as a consequence of ACTH stimulation (6–8), hypophysectomy (9–11), and cortisone administration (11, 12) have been the subject of several electron microscope studies. However, no systematic investigation of the stimulated zona glomerulosa at the fine structural level has been published. It was therefore felt that an ultrastructural study of the zona glomerulosa after stimulation might help to elucidate some of the structural phenomena associated with steroid biosynthesis. These experiments reveal additional fine structural features which are deemed important in relation to steroid biosynthesis.

MATERIALS AND METHODS

Stimulation of the zona glomerulosa of a group of 15 male Columbia Sherman rats weighing 100 gm was achieved by feeding the animals a sodium-deficient diet[1] (13) for periods of time ranging up to 1 month. The rats were sacrificed after intervals of 2, 7, 14, 21, and 28 days of salt restriction. Three of these animals, as well as a control rat maintained on Purina Rat-Mouse Chow, were sacrificed at each of these periods.

Adequate preservation of the fine structure of the adrenal cortex proved to be a problem. However, after employing numerous methods of fixation, the following procedure finally afforded excellent preser-

[1] Sodium-deficient test diet, Nutritional Biochemicals Corporation, Cleveland, Ohio.

vation of cell structure. The left adrenal gland of all the animals was promptly exposed, under Nembutal anesthesia, and continuously flooded *in situ* with cold 2 per cent buffered (pH 7.4 Veronal-acetate) osmium tetroxide with added sucrose for 30 minutes. These glands were then excised and fixed *in toto* in fresh osmium tetroxide for an additional 4 hours, following which they were dehydrated in a graded series of acetones. Small pieces of superficial cortical tissue including capsule were carefully cut from the glands under a dissecting microscope during the final dehydration steps and then infiltrated in Araldite at 5°C for 2 to 3 days. The specimens were oriented so that the sections included the entire thickness of the zona glomerulosa as well as the adjacent zona fasciculata and capsule.

Two-μ thick sections of the Araldite-embedded material were examined under a phase-contrast microscope, and thin sections stained with lead hydroxide (14) were examined under a Siemens Elmiskop I microscope. Paraffin-embedded and frozen sections were prepared from the formalin-fixed contralateral adrenal gland of each animal. These sections were stained with hematoxylin and eosin, and Sudan IV (15), respectively, and examined by ordinary light microscopy.

RESULTS

Light microscopy reveals progressive enlargement of the zona glomerulosa over the course of the experiment. After 1 month of salt restriction the zona glomerulosa is approximately 2 to 3 times greater in width than that of the control animals (Figs. 1 and 2). In the control animals numerous large sudanophilic droplets are present throughout the cells of the zona glomerulosa. Two-μ thick Araldite sections of tissue from the control animals examined under the phase-contrast microscope disclose occasional cells which have intracytoplasmic droplets of high density (Fig. 1). During the course of the experiment there is a decrease in the size and number of the sudanophilic droplets. At the same time, phase-contrast microscopy discloses increased numbers of the dense cytoplasmic droplets (Fig. 2).

The fine structure of the unstimulated zona glomerulosa is in general similar to that described by previous workers (1–4, 12). Two cell types are present (Fig. 3). The first of these, termed a light cell, has a cytoplasmic ground substance of low electron opacity and contains numerous large cytoplasmic droplets which in some cases occupy the major portion of the cytoplasm (Figs. 3 and 4). The contents of the droplets, which are homogeneous and of low electron opacity, are delimited from the adjacent cytoplasm by a single membrane. The droplets measure up to 2.0 μ in diameter, have round to irregular profiles, and are often in very close proximity to mitochondria (Figs. 4 and 5). The mitochondria are relatively uniform in size, measure approximately 0.6 μ in diameter, and have oval to circular outlines. These mitochondria are demarcated by a continuous double membrane whose inner layer is projected into the interior of the organelles in the form of tubules rather than lamellae (Figs. 5 and 6), (1–3, 5–9, 16–18). The intramitochondrial tubules pursue a somewhat irregular course and have variable diameters.

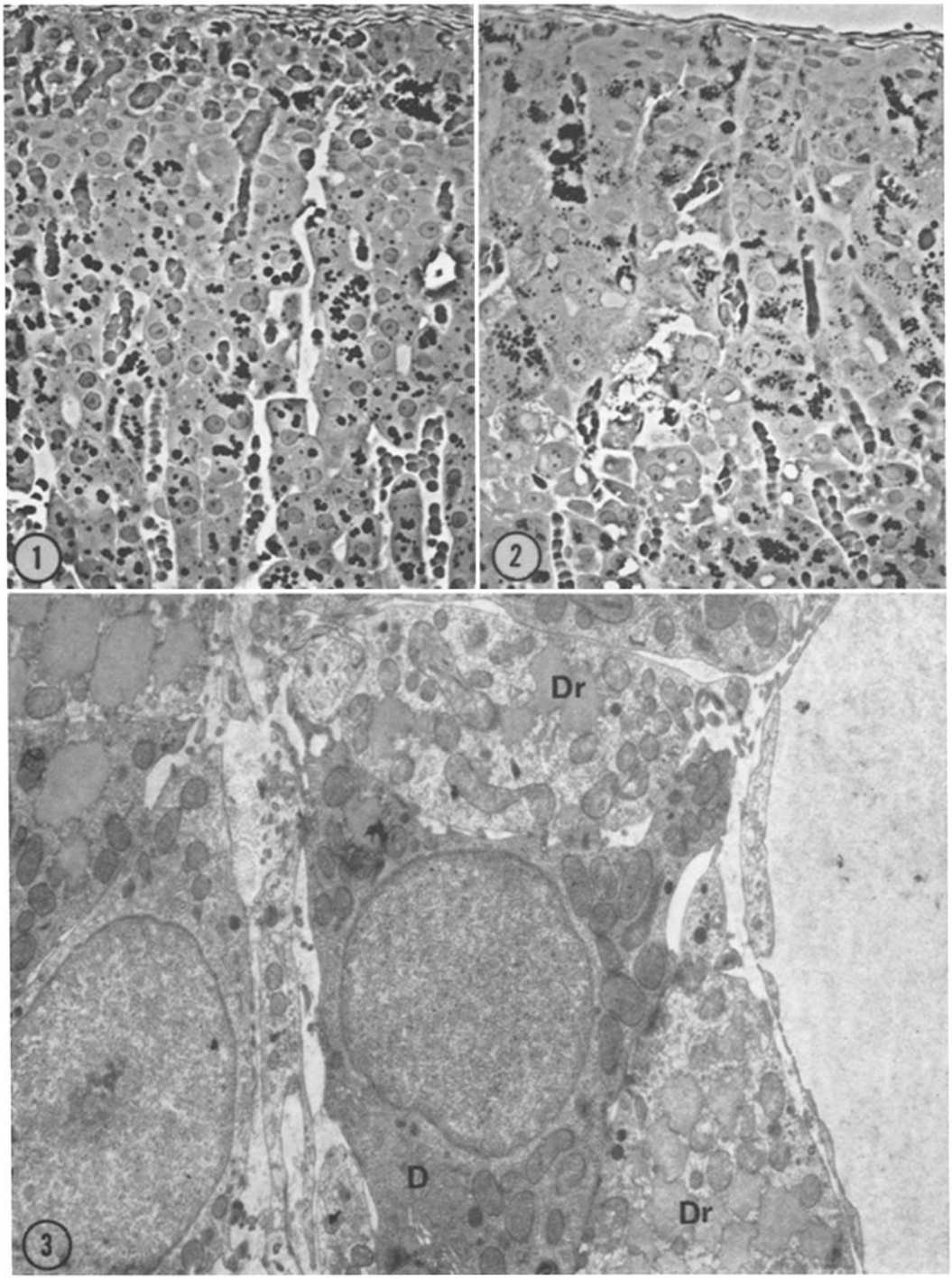
Present are a prominent Golgi complex (Fig. 6) and a moderate amount of smooth-surfaced endo-

FIGURES 1 and 2 are phase-contrast micrographs of 2-μ thick, Araldite-embedded sections of adrenal glands, while the remaining figures are electron micrographs of the zona glomerulosa. Figs. 1, 3 to 6, and 12 are from control (unstimulated) rat adrenal glands. Figs. 2, 7 to 11, and 13 to 25 are from stimulated rat adrenal glands.

FIGURE 1 This is a phase-contrast micrograph of a control adrenal gland. There are relatively few dense intracytoplasmic droplets in the zona glomerulosa as compared with the zona fasciculata. × 400.

FIGURE 2 Note the increased thickness of the zona glomerulosa following 4 weeks of sodium deprivation. The nuclei of the hyperplastic cells in the zona glomerulosa are more widely separated than those of the control gland (see Fig. 1). Increased numbers of cytoplasmic droplets are present in this portion of the adrenal gland. × 400.

FIGURE 3 Electron micrograph of normal zona glomerulosa showing a dark cell (*D*) which contains fewer cytoplasmic droplets (*Dr*) than the light cell. × 10,000.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plasmic reticulum, which usually appears as round to vesicular profiles (Figs. 5 and 6). The irregularities on the surfaces of the droplets described above often consist of small outpouchings into the cytoplasm which are similar in diameter to the tubules of the smooth-surfaced endoplasmic reticulum (Fig. 5). The contents of these outpouchings, however, are of higher electron opacity than the material within the smooth-surfaced endoplasmic reticulum. Small groupings of rough-surfaced endoplasmic reticulum are noted as well as numerous non - membrane – associated ribosomes which are dispersed throughout the cell (Figs. 4 and 5). The second cell type, or dark cell, is smaller than the light cell and has a cytoplasm which is generally more electron-opaque than that of the latter (Fig. 3). The cytoplasmic droplets are fewer in number and smaller in the dark cells than in the light cells.

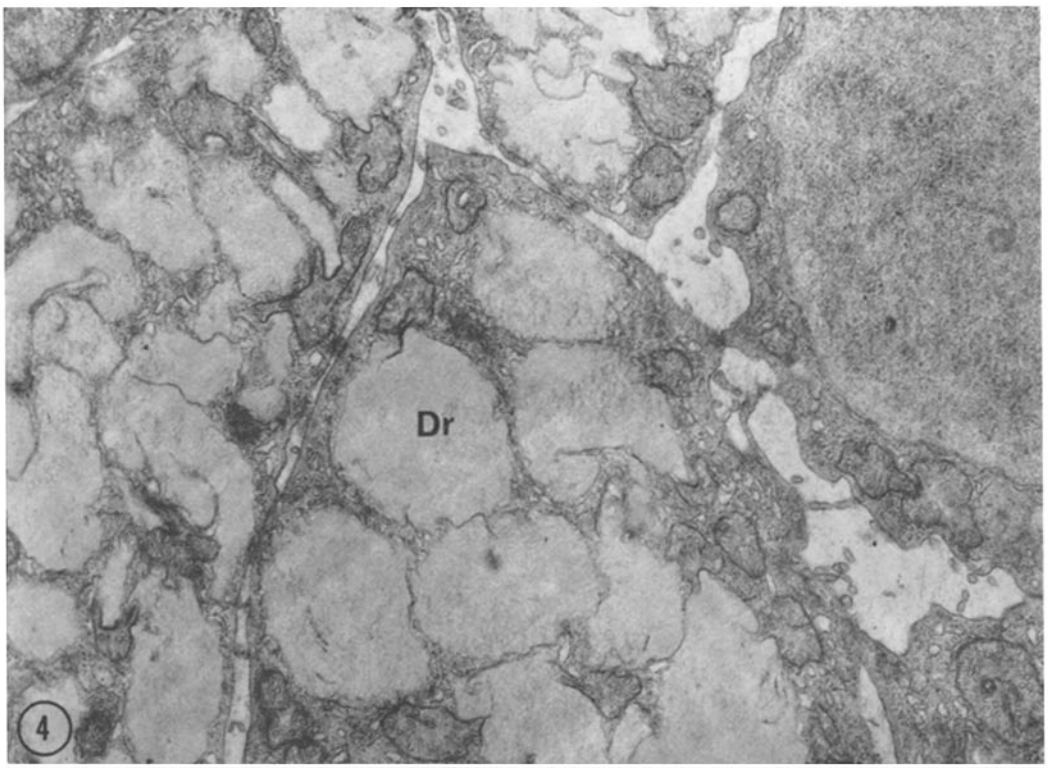
Dark cells are decreased in number after 2 days of sodium deprivation and have disappeared by the 7th day of sodium restriction. The light cells, which now comprise the entire zona glomerulosa in animals fed the experimental diet, display a series of progressive cytoplasmic changes. These alterations are more pronounced the longer the interval of salt restriction, and affect the cytoplasmic droplets, mitochondria, and smooth-surfaced endoplasmic reticulum as well as other structures.

In general, there is a progressive decrease in the number of cytoplasmic droplets during the experiment. After 2 days an occasional zona glomerulosa cell is devoid of these droplets (Fig. 7). After 21 days, more numerous cells lacking these structures are noted (Figs. 18, 21, and 23). In addition to a decrease in their number the droplets are smaller in diameter and have more regular profiles (Figs. 8, 10, 17 to 19, and 23), over the course of the experiment, when compared with the control material (Figs. 3 to 5). The cellular area occupied by these droplets has been quantitated by the method described by Loud (19) utilizing a counting frame with 12 lines that are 10 cm long. In the control animals, the cytoplasmic droplets occupy 11.6 per cent of the cell cross-sectional area; in the animals placed on salt restriction for 28 days, these droplets occupy 5.6 per cent of the cell cross-sectional area.

The mitochondria also exhibit striking changes. Numerous, greatly enlarged, elongated mitochondria measuring up to 4 μ in length appear to be present with increasing frequency towards the end of the experiment. Such mitochondria exhibit regular arrays of lamellae which usually but not invariably run parallel to the long axis of the mitochondria (Figs. 8 to 12, and 14 to 17). Different planes of section reveal that these apparent lamellae are formed by hexagonal arrays of parallel tubules (Figs. 12 and 13). The tubules measure 225 A in diameter and have a center-to-center spacing of approximately 300 A. These latter tubules differ from the other mitochondrial tubules in several respects. They run a straight course parallel to each other, their limiting membranes are thicker and more electron-opaque, and they have a uniform diameter which is smaller than that of the typical intramitochondrial tubules (Figs. 12, 13, and 15). In many instances continuity between the two types of tubules can be demonstrated (Figs. 14 and 15). Frequently, mitochondria which contain these linear arrays of tubules have large discontinuities in their double-limiting membranes (Figs. 8, 10, 11, and 15 to 17). The parallel arrays of tubules often extend from within the mitochondria through these discontinuities into the cytoplasm where they are in close apposition to the smooth-surfaced endoplasmic reticulum (Figs. 11, 16, and 17). Mitochondria showing similar features are encountered in the unstimulated cells of the zona glomerulosa (Fig.

FIGURE 4 Another electron micrograph of unstimulated zona glomerulosa discloses irregular profiles of the cytoplasmic droplets (*Dr*) which are often in close apposition to mitochondria. Small aggregates of rough surfaced endoplasmic reticulum as well as dispersed ribosomes are present. × 15,000.

FIGURE 5 Similar to Fig. 4. The intimate relationship between mitochondria and the irregularly contoured lipid droplets (*Dr*) is shown. There is a relative abundance of smooth-surfaced endoplasmic reticulum. Note the non-membrane–associated ribosomes, occasional cisternae of the granular endoplasmic reticulum, and the intramitochondrial tubules. × 54,000.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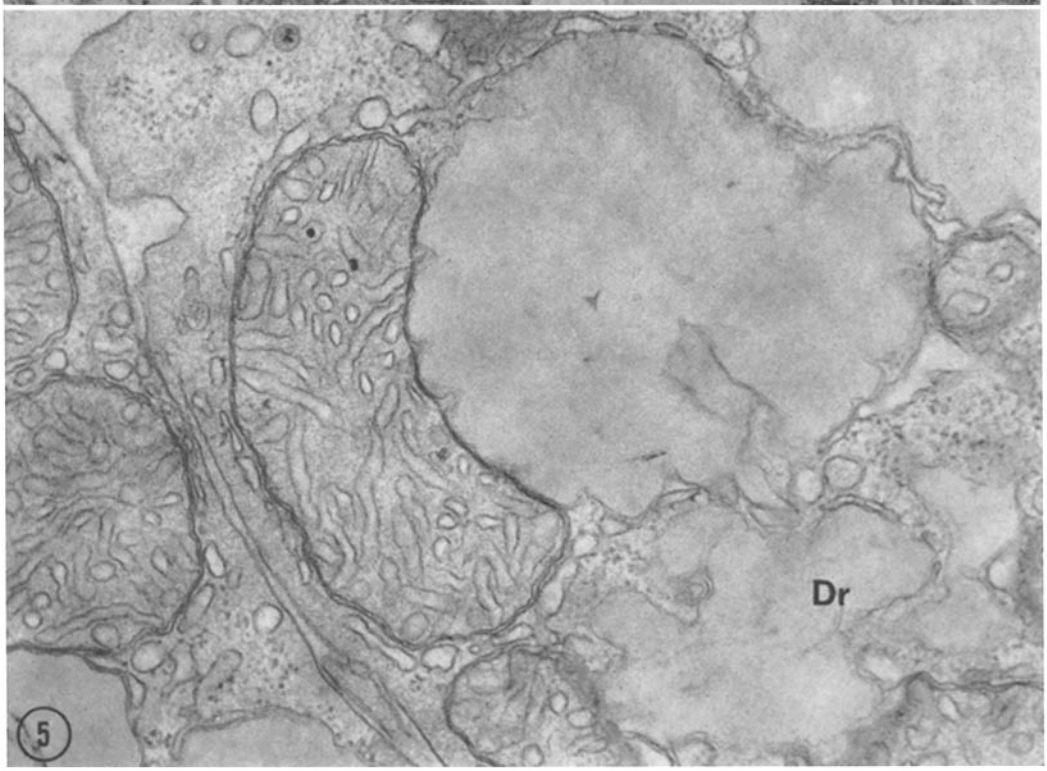
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12). While these mitochondria appear to be increased in number in the sodium-deficient animals, statistical analysis does not confirm this impression. The number of giant mitochondria per cell was evaluated in a large number of electron micrographs and no significant statistical difference was found in the control group as compared with the animals placed on a sodium-restricted diet for 28 days.

A prominent mitochondrial change occurs in the last 2 weeks of the experiment and consists of the accumulation of amorphous deposits within many mitochondria (Figs. 8, 18 to 20, and 23). The intramitochondrial deposits are not seen in the zona glomerulosa cells of either control animals or of animals maintained on a sodium-restricted diet for less than 2 weeks. By the 28th day of the experiment, quantitative evaluation of these intramitochondrial deposits shows on the average that there is at least one such structure per zona glomerulosa cell. These deposits, which vary considerably in size and number within the mitochondria, are more electron-opaque than the contents of the cytoplasmic droplets described above. Mitochondria containing large amounts of this material do not exhibit the parallel tubular arrays and contain relatively few intramitochondrial tubules of the other variety.

Increased amounts of smooth-surfaced endoplasmic reticulum and/or Golgi apparatus is another feature of the cells of the stimulated zona glomerulosa (Figs. 7, 10, and 21). This change is seen throughout the entire period of salt deprivation. Complex arrangements of tubules or cisternae can be seen in varying planes of section (Fig. 21). Parallel arrays of lamellae which partially or completely encircle small portions of cytoplasm are present (Fig. 22). These structures resemble so called autophagosomes (20, 21).

Cytoplasmic bodies that are in part considerably more electron-opaque than the cytoplasmic droplets or amorphous deposits within mitochondria are noted with increasing frequency towards the latter phases of the experiment. These bodies, in common with similar bodies that are usually described as either lysosomes or microbodies (22), display a variety of structures including a distinct limiting membrane, variations in electron opacity and granularity, and arrays of lamellae (Figs. 10, 11, 18, and 22 to 24). These dense bodies are found less frequently in the cells of the unstimulated zona glomerulosa (Fig. 6).

In the last week of the experiment large, irregular, non-membrane-limited and highly electron-opaque cytoplasmic masses are seen (Fig. 25). The electron opacity and contours of these masses resemble those of lipid accumulations in other cell types. These highly electron-opaque masses are not found in the glomerulosa cells of control animals. Statistical analysis, at the end of 28 days of salt restriction, reveals an average of one electron-opaque mass per cell.

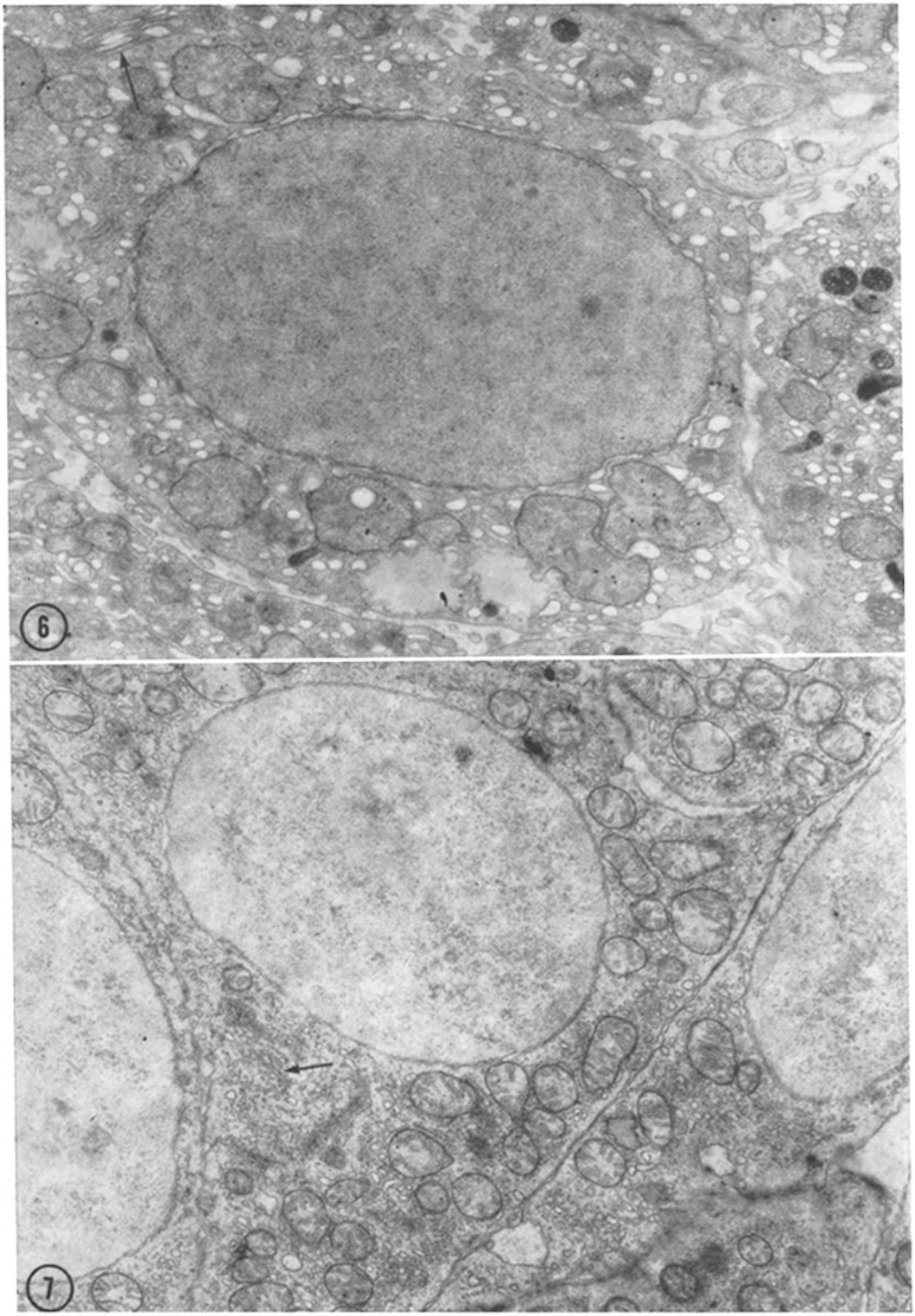
The cell surface membranes in the stimulated glands become increasingly irregular and exhibit numerous microvilli. Large cytoplasmic vacuoles and deep surface membrane invaginations, which surround granular material similar to that seen in the extracellular space elsewhere, are noted (Figs. 18 and 23).

DISCUSSION

It has been well established that the zona glomerulosa, which is independent of pituitary control, is stimulated by salt restriction. Under these conditions, the zona glomerulosa enlarges and synthesizes increased amounts of aldosterone (13, 23–30). The absence of dark cells in the stimulated zona glomerulosa suggests that these relatively inactive or reserve cells are transformed into light cells which have a greater capacity for steroid biosynthesis. Christensen and Fawcett have suggested that the variations in cytoplasmic density

FIGURE 6 Numerous circular and oval profiles representing smooth-surfaced endoplasmic reticulum are present in these unstimulated zona glomerulosa cells. A Golgi complex is indicated by the arrow. A number of dense bodies are also seen near the right margin of the figure. × 13,500.

FIGURE 7 Electron micrograph from an animal maintained on a salt-restricted diet for two days shows prominent Golgi complexes (arrow) as well as the absence of cytoplasmic droplets. × 11,500.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of the interstitial cells of the testis may be related to hormone storage by these cells (31). Changes in the cytoplasmic density of zona fasciculata cells have also been observed after hypophysectomy and ACTH stimulation (7, 9). Lever, however, has noted the presence of dark cells in the stimulated zona glomerulosa (9) which is at variance with the present observations. This difference may possibly be ascribed to differences in preparative techniques or to the method utilized for stimulating the zona glomerulosa.

The cytoplasmic droplets of low electron opacity, which are extremely numerous in the unstimulated glands, are not apparent with phase-contrast microscope techniques. These droplets, which decrease in size and number in the stimulated glands, may consist of cholesterol. This suggestion is supported by histochemical and biochemical data which show not only the presence of large quantities of cholesterol and its esters within the cytoplasm of adrenal cortical cells but also a decrease in cholesterol content in stimulated adrenal glands (13, 32–40). The low electron opacity of these droplets could be explained by the non-polar properties of cholesterol which therefore binds relatively little osmium tetroxide. Cholesterol esters, which constitute 25 per cent of the cholesterol present in the microsomal and supernatant fractions of the adrenal gland (39), may further augment the non-polar nature of these cytoplasmic droplets.

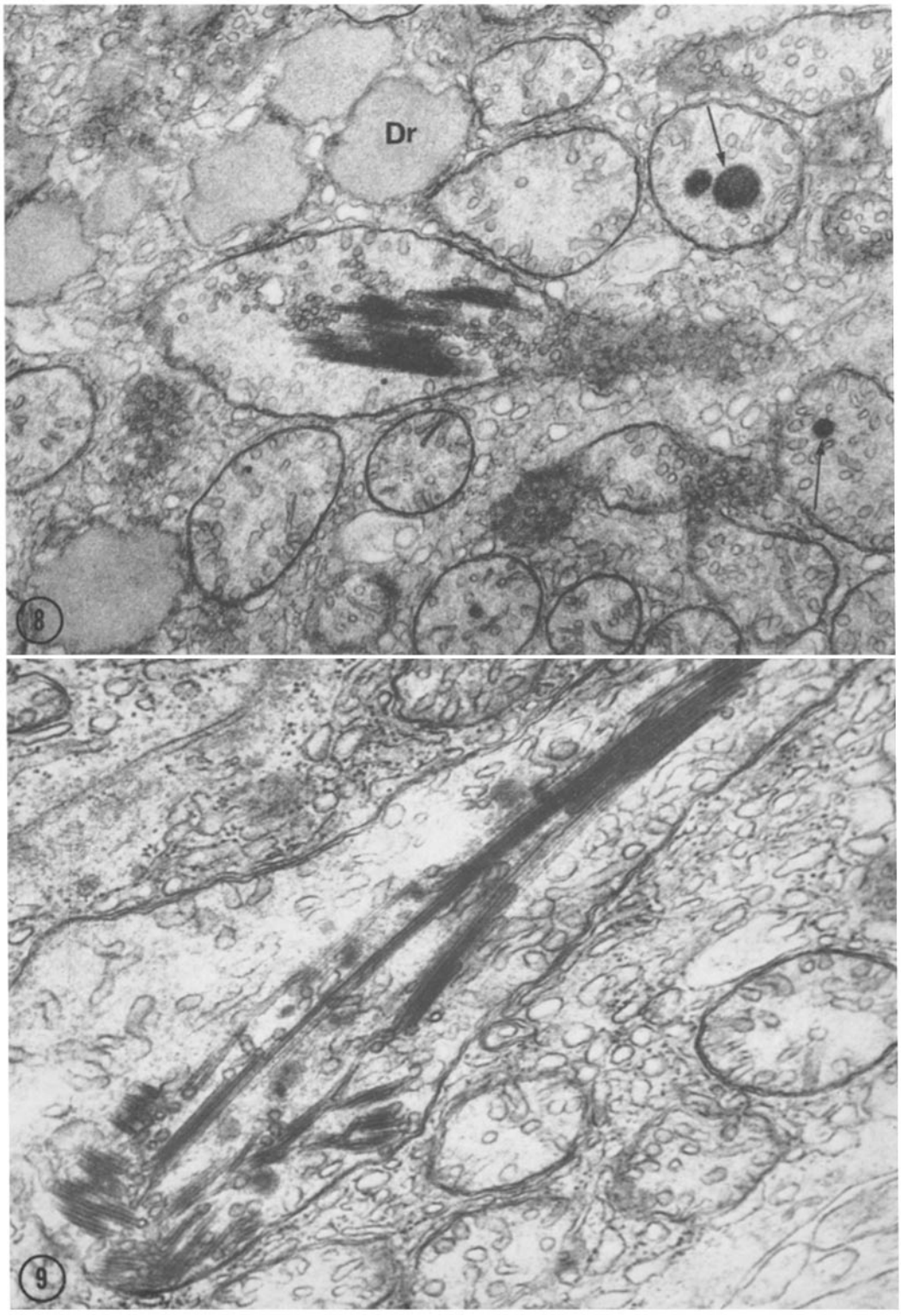
The biosynthesis of aldosterone from its major precursor cholesterol is thought to occur through a series of steps in which different cell fractions participate (41). The primary degradation of the cholesterol side chain to form Δ^5-pregnenolone by a desmolase occurs in the mitochondrial fraction (37, 40). Δ^5-pregnenolone is oxidized to progesterone by the enzyme 3-β-dehydrogenase which has been localized, for the most part, to the microsomes of the cell (42). The 21-carbon methyl group is then hydroxylated, by means of 21-hydroxylase present in the microsomes, to 11-desoxycorticosterone (42, 43). Subsequent hydroxylations by means of the enzymes 11-β-hydroxylase and 18-hydroxylase, both of which are in the mitochondrial fraction (35, 42, 44–47), result in the formation of corticosterone, 18-hydroxycorticosterone, and the final product aldosterone. These enzymatic reactions require either nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP) as well as molecular oxygen (42).

This series of steps in aldosterone biosynthesis permits a number of speculations regarding the functional significance of the various cytoplasmic structures observed in this study. The close proximity between mitochondria and the extramitochondrial droplets, which may be composed of cholesterol, would facilitate the transport of cholesterol to the mitochondria, where the primary degradation of its side chain to form Δ^5-pregnenolone occurs.

Giant mitochondria containing regularly packed tubules are noted in both the control and stimulated zona glomerulosa cells. These tubules, as previously described, are definitely continuous with but differ in dimensions and other morphological features from the more usual mitochondrial tubules. They often extend from within the mitochondria into the adjacent cytoplasm, where they are in intimate contact with the smooth-surfaced endoplasmic reticulum. Previous workers have described the abundance of smooth-surfaced endoplasmic reticulum in the cells of various steroid-secreting organs (1, 3, 5, 7, 11, 12, 31, 48–52). This is emphasized by the presence, in this study, of increased quantities of smooth-surfaced endoplasmic reticulum and/or Golgi complexes in the hyperplastic zona glomerulosa relative to the control adrenal glands. It is known that the endoplasmic reticulum plays an important role in steroid biosynthesis, inasmuch as 3-β-dehydrogenase and 21-hydroxylase, which catalyze steps

FIGURE 8 A large mitochondrion whose surface membranes appear discontinuous at one end contains parallel lamellae. Several other mitochondria contain deposits (arrows) which are more electron-opaque than the cytoplasmic droplets (*Dr*). 28 days of salt restriction. × 28,500.

FIGURE 9 A giant mitochondrion containing parallel lamellae oriented in several directions is depicted. 3 weeks of salt restriction. × 50,000.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resulting in the conversion of Δ^5-pregnenolone to 11-desoxycorticosterone, are associated with the microsomic fraction (42, 43, 53). The regularly packed tubular arrays found in the mitochondria may provide the structural framework for these sequential steps utilized in the conversion of cholesterol to 11-desoxycorticosterone which, as mentioned above, are thought to occur first in the mitochondria and then in the microsomes. They might also provide the necessary link between the extramitochondrial dehydrogenase and hydroxylases with NAD or NADP and the electron transport arrays which are located along the cristae of most mitochondria (54–62). The membranes of the mitochondrial-to-cytoplasmic tubular system might also play a role in the transport of the more hydrophobic steroid intermediates from one portion of the cell to another. While the major portion of 3-β-dehydrogenase activity is confined to the microsomal fraction, 10 per cent of this enzymatic activity is firmly associated with the mitochondrial fraction (39, 42). This affords additional support for both a structural and functional link between mitochondria and other cytoplasmic organelles.

The significance of these rather unique mitochondria cannot be definitely assessed at this time. However, they must have general implications in terms of steroid biosynthesis, inasmuch as some of their structural features have been observed by various investigators in other steroid-secreting tissues including the zona fasciculata and reticularis, corpus luteum, and theca interna of the graafian follicle (1–3, 5–9, 11, 16–18, 31, 50, 63). However, the continuity between mitochondrial tubules and cytoplasm has only recently been demonstrated, possibly due to improved tissue preparative techniques (64, 65).

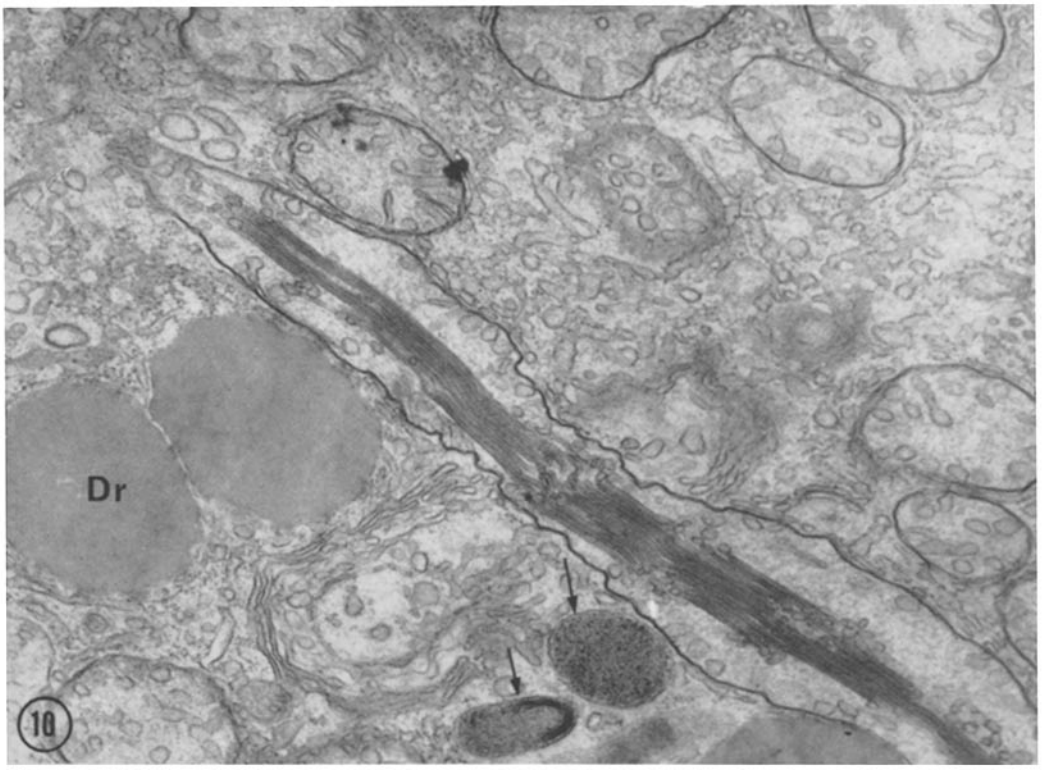
A prominent mitochondrial alteration consists of the accumulation of material within mitochondria which is more electron-opaque than that of the cytoplasmic droplets. Similar intramitochondrial deposits in steroid-secreting tissues have been observed (1, 2, 7, 9, 10, 16, 31, 65, 66), and several workers have regarded these deposits as intramitochondrial lipid accumulation (1, 7, 9, 65). The final steps in the synthesis of aldosterone from desoxycorticosterone involve the 11-β- and 18-hydroxylations which occur within the mitochondria (39, 42, 44–47). The intramitochondrial deposits could represent the intermediates corticosterone and 18-hydroxycorticosterone and the final product aldosterone. The relatively greater osmiophilia of this material as compared with that of the cytoplasmic droplets may be due to the more polar nature of these latter steroids.

Morphologically, at least two distinct populations of mitochondria are present. The suggestion that one is concerned with the earlier biosynthetic steps and the other with the later phases of aldosterone formation remains to be established by means of *in vitro* biochemical studies of mitochondrial and other cell fractions. It is readily apparent that the interpretation of complex cytoplasmic changes requires studies utilizing a variety of techniques in addition to purely morphological ones.

The manner in which aldosterone is released from the mitochondria and secreted by the cells is unsettled. The extremely electron-opaque nonmembrane–limited cytoplasmic inclusions may be composed of aldosterone released into the cytoplasm. The prominent ruffling of the cell surface as well as the deep invaginations of the stimulated zona glomerulosa cells may be related to the mechanism of hormone release by these cells as previously suggested by Carr (8). The significance of the increased number of lysosome-like dense bodies (or microbodies) and structures resembling autophagosomes is not clear. The deeply osmiophilic inclusions seen under the phase-contrast microscope which are more prominent in the

FIGURE 10 Similar to Fig. 9. The interior of the giant mitochondrion appears to be continuous with the cytoplasm. A Golgi complex is apparent between 2 dense bodies (arrows) and 2 cytoplasmic droplets (*Dr*). The former possess granular and lamellar structures as well as a limiting membrane. 4 weeks of sodium deprivation. × 36,000.

FIGURE 11 The parallel intramitochondrial lamellae appear to extend from within the mitochondrion or mitochondria into the cytoplasm. Several dense bodies are noted (arrows). 4 weeks of salt deprivation. × 38,000.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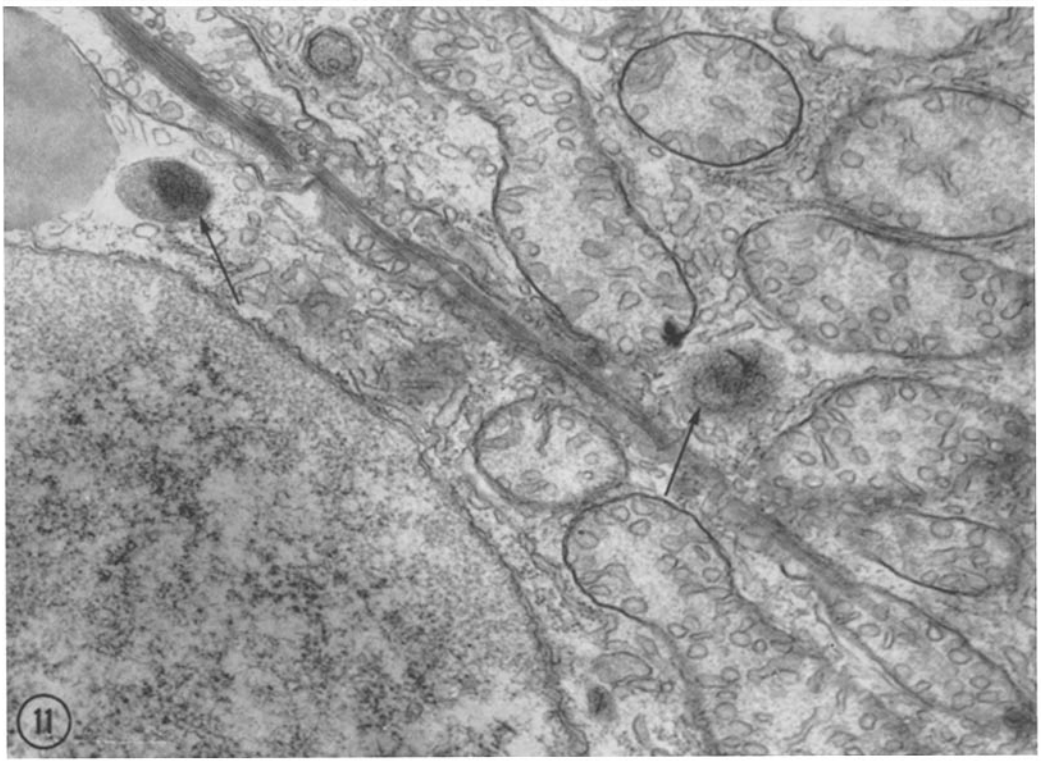
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stimulated zona glomerulosa may represent the extremely electron-opaque, non-membrane-limited cytoplasmic inclusions as well as the lysosome-like bodies.

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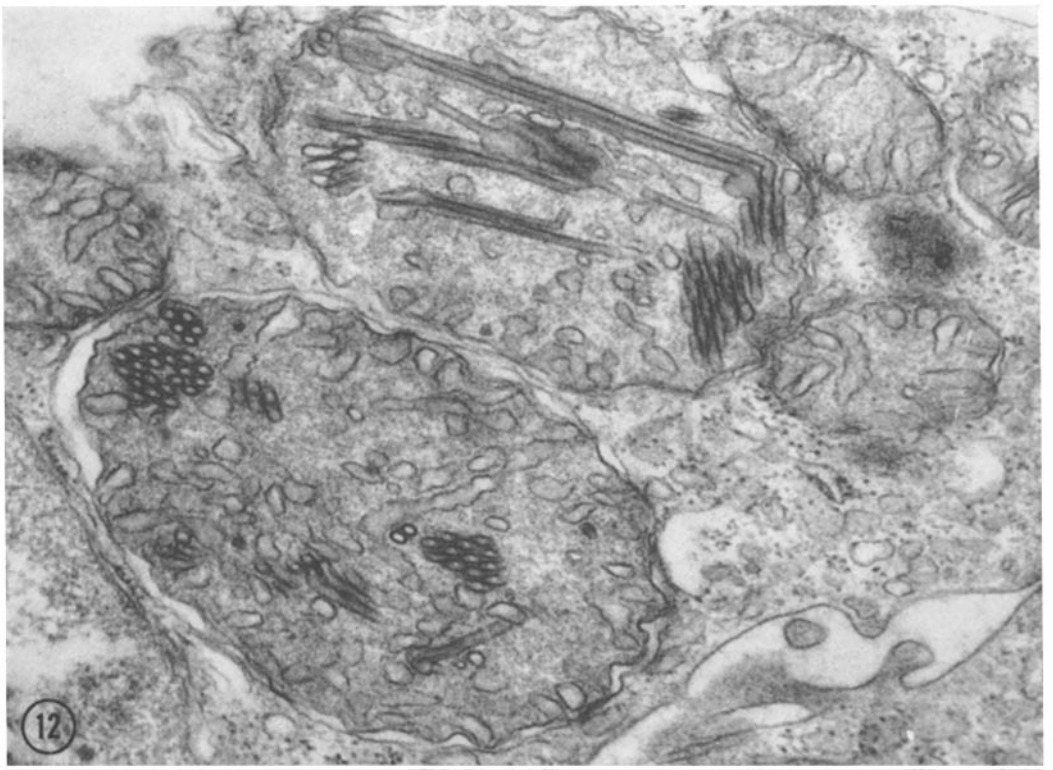
FIGURE 12 Two mitochondria contain parallel arrays of tubules cut in varying planes ranging from longitudinal to transverse. These latter tubules differ from the more usual mitochondrial tubules as regards the electron opacity and thickness of their walls and the diameters of their lumina (control zona glomerulosa). × 50,000.

FIGURE 13 This mitochondrion contains a parallel array of tubules in transverse section. These tubules exhibit hexagonal packing. The differences in structure between the two types of intramitochondrial tubules are readily apparent. 14 days of sodium deprivation. × 56,000.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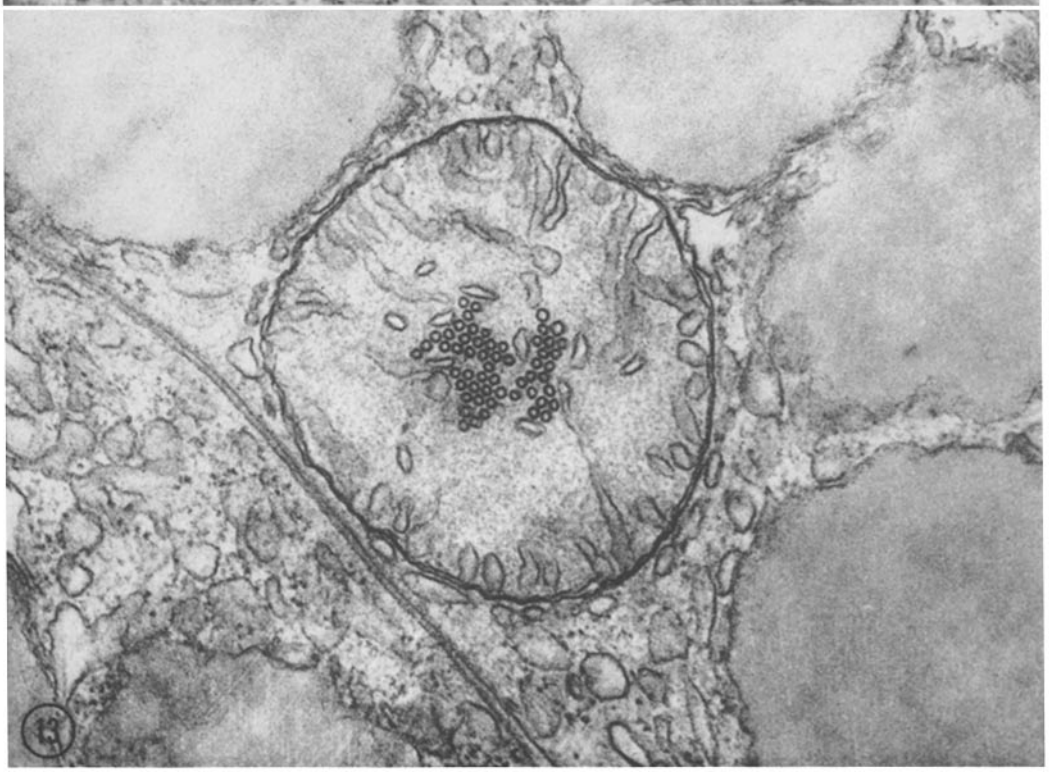
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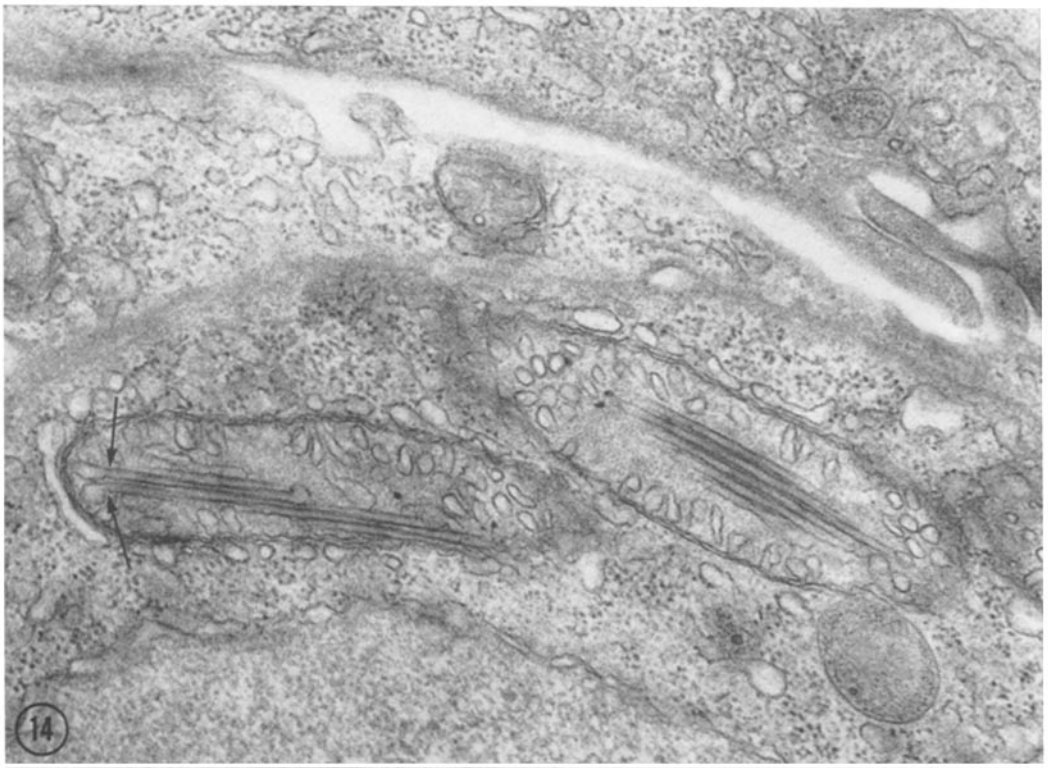
FIGURE 14 Continuity between the parallel tubules and the more usual mitochondrial tubules is shown (arrows). A dense body is noted in the lower right portion of the figure. 1 week of salt restriction. × 52,000.

FIGURE 15 This figure also demonstrates continuity between the two types of mitochondrial tubules (double arrows). The enclosing mitochondrial membrane system is discontinuous at one end of the mitochondria. At the margins of this discontinuity there is fusion of the inner and outer limiting mitochondrial membranes (single arrows). 23 days of sodium restriction. × 90,000.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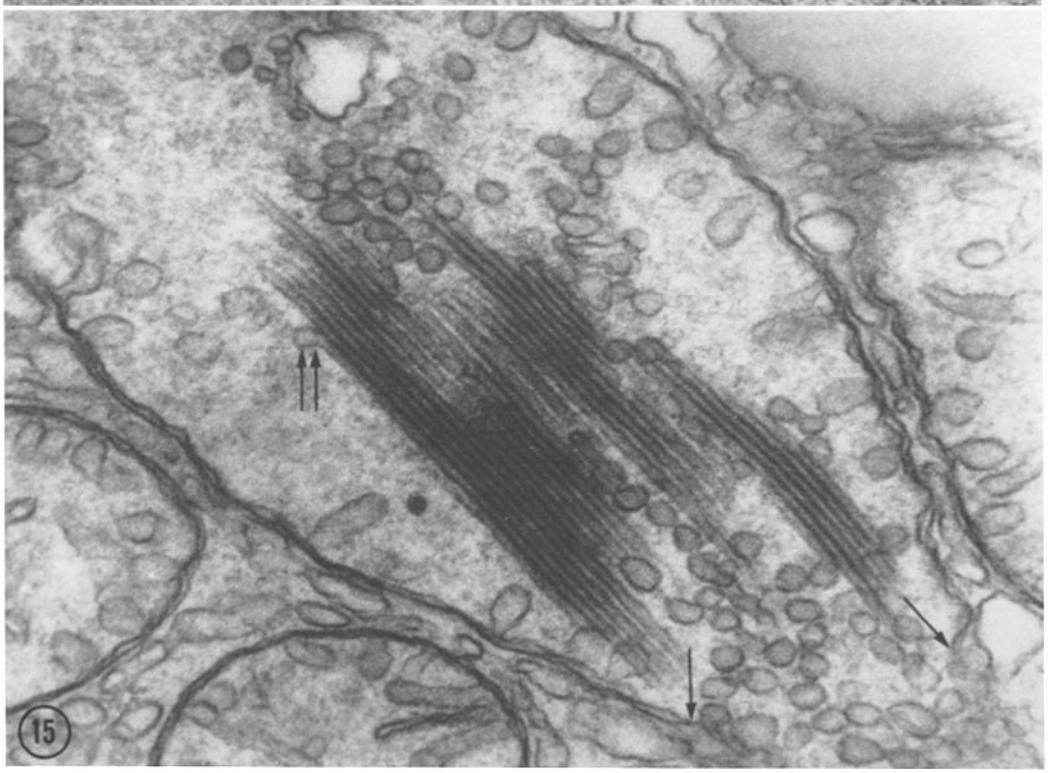
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FIGURE 16 Parallel intramitochondrial tubules project through the open end of a mitochondrion into the adjacent cytoplasm. 3 weeks of salt restriction. × 60,000.

FIGURE 17 Similar to preceding figure. 7 days of sodium deprivation. × 60,000.

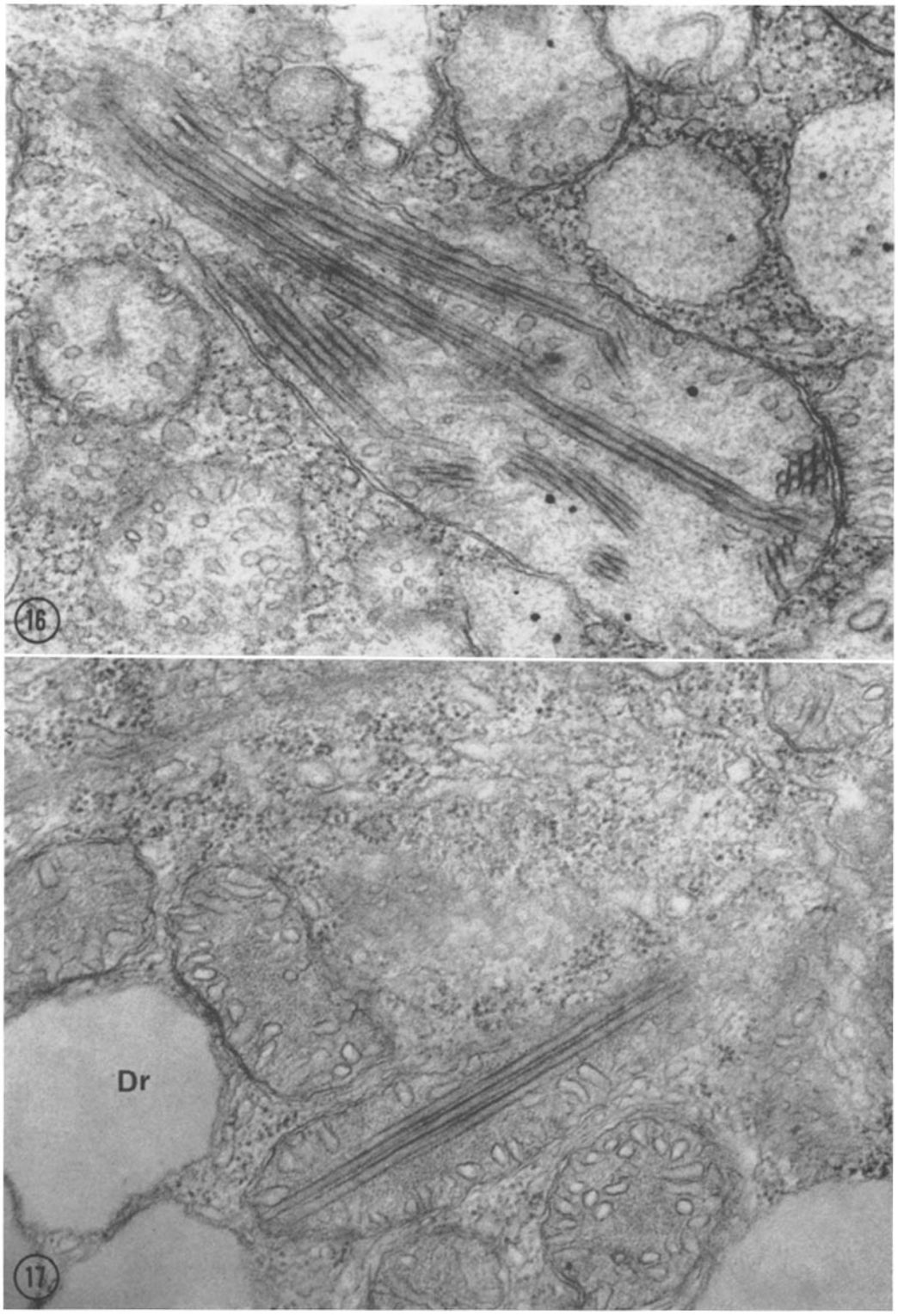
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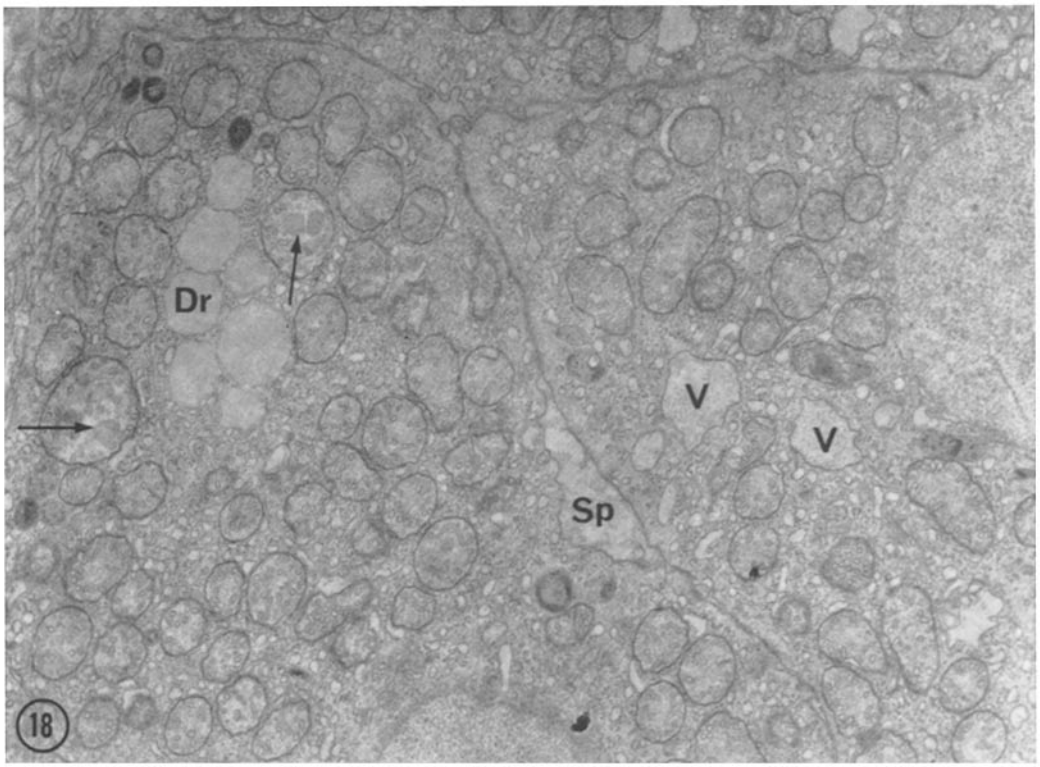
FIGURE 18 Relatively few cytoplasmic droplets (*Dr*) are present. Deposits are present within a few mitochondria (arrows). The cell on the right contains two irregular membrane-limited vacuoles enclosing granular material (*V*) similar to that present in the adjacent intercellular space (*Sp*). A number of dense bodies are present in the upper left portion of the figure. 3 weeks of salt restriction. × 12,500.

FIGURE 19 Numerous mitochondria contain intramitochondrial deposits (arrows) which are of greater electron opacity than the cytoplasmic droplets (*Dr*). These mitochondria possess relatively few tubules. 3 weeks of sodium deprivation. × 18,000.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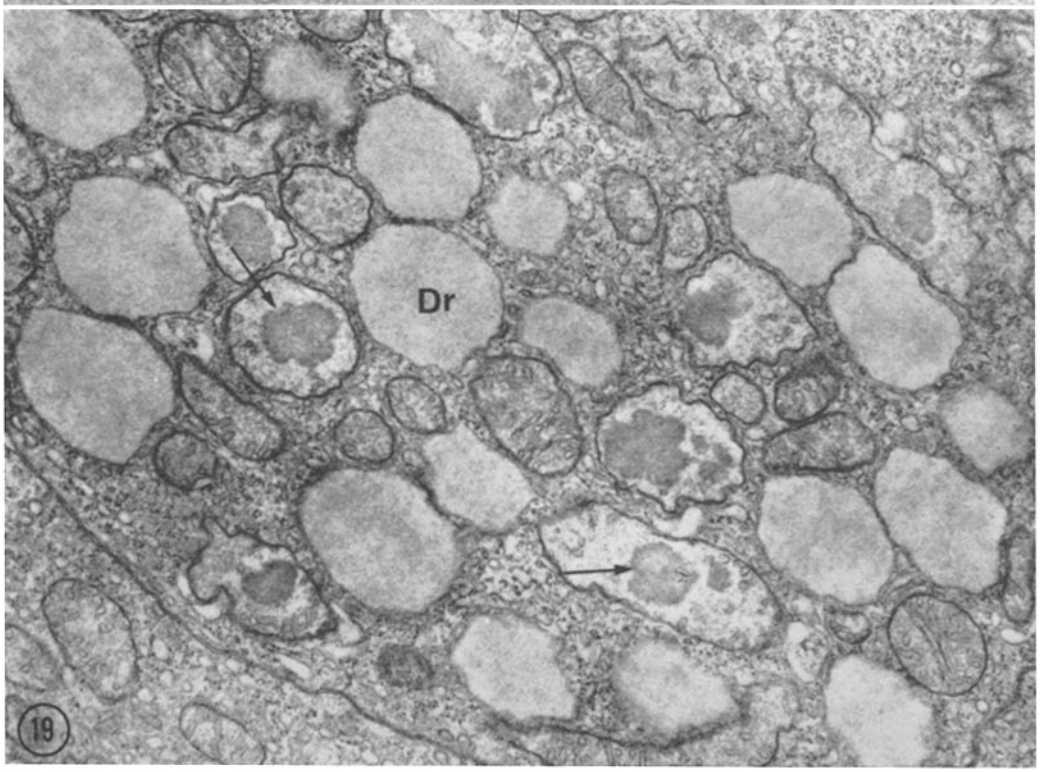
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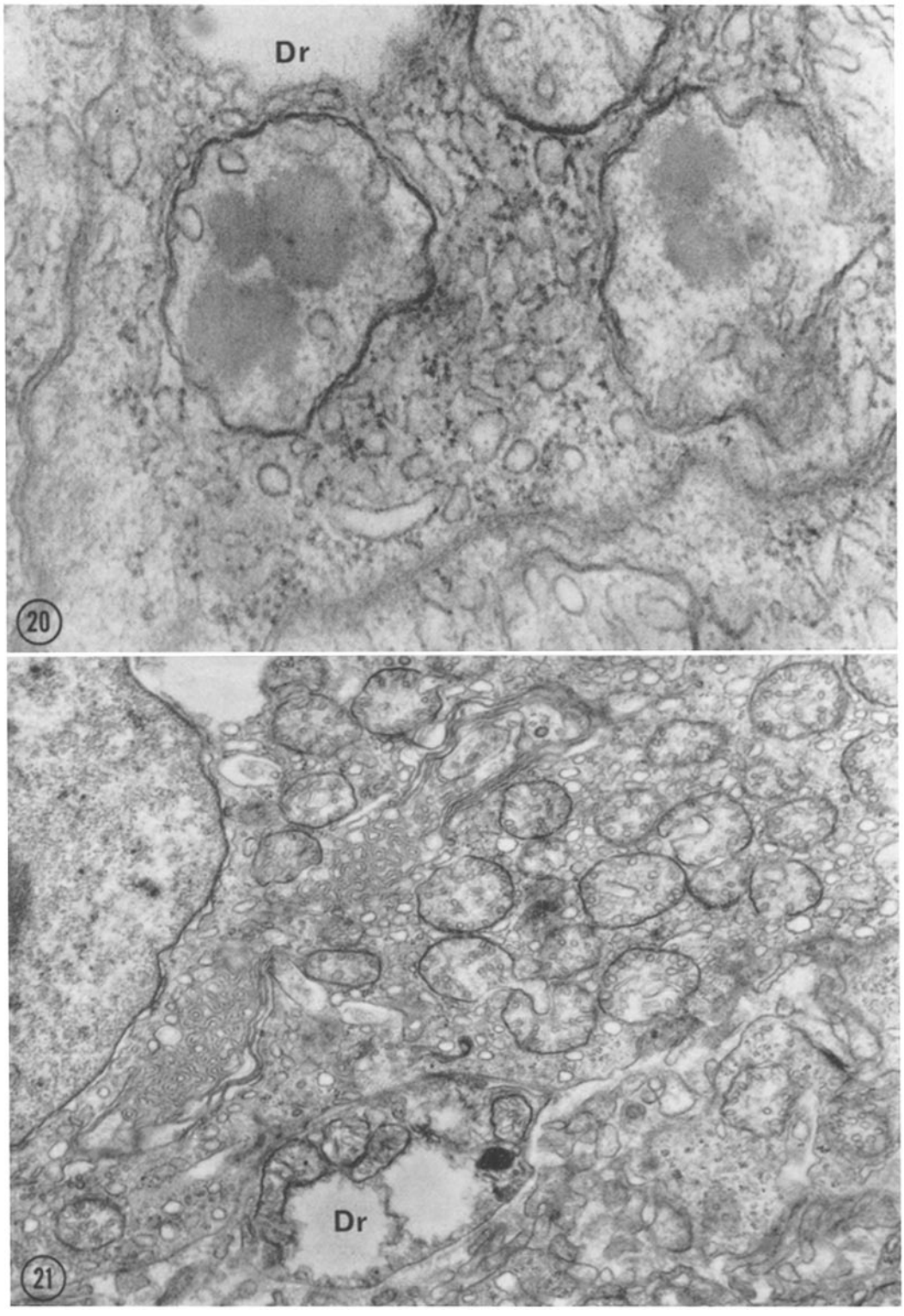
FIGURE 20 Similar to Fig. 19. Note the intramitochondrial deposits. 21 days of salt restriction. × 80,000.

FIGURE 21 Complex arrays of tubules cut in varying planes can be seen. 4 weeks of sodium deprivation. × 20,000.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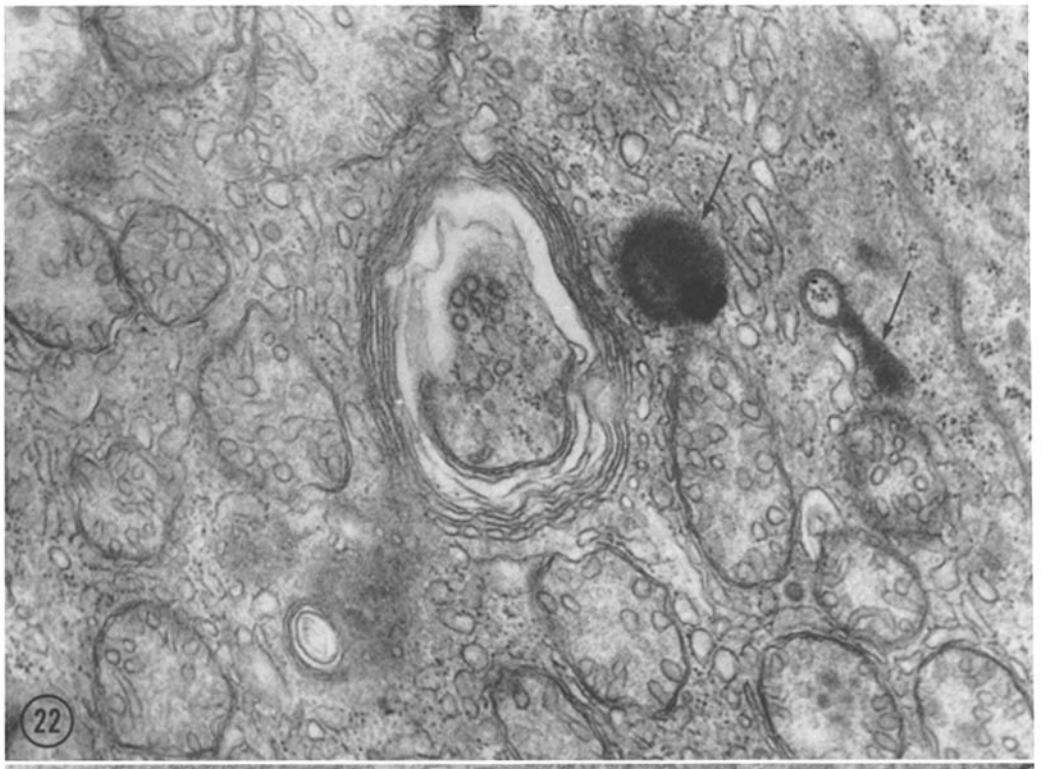
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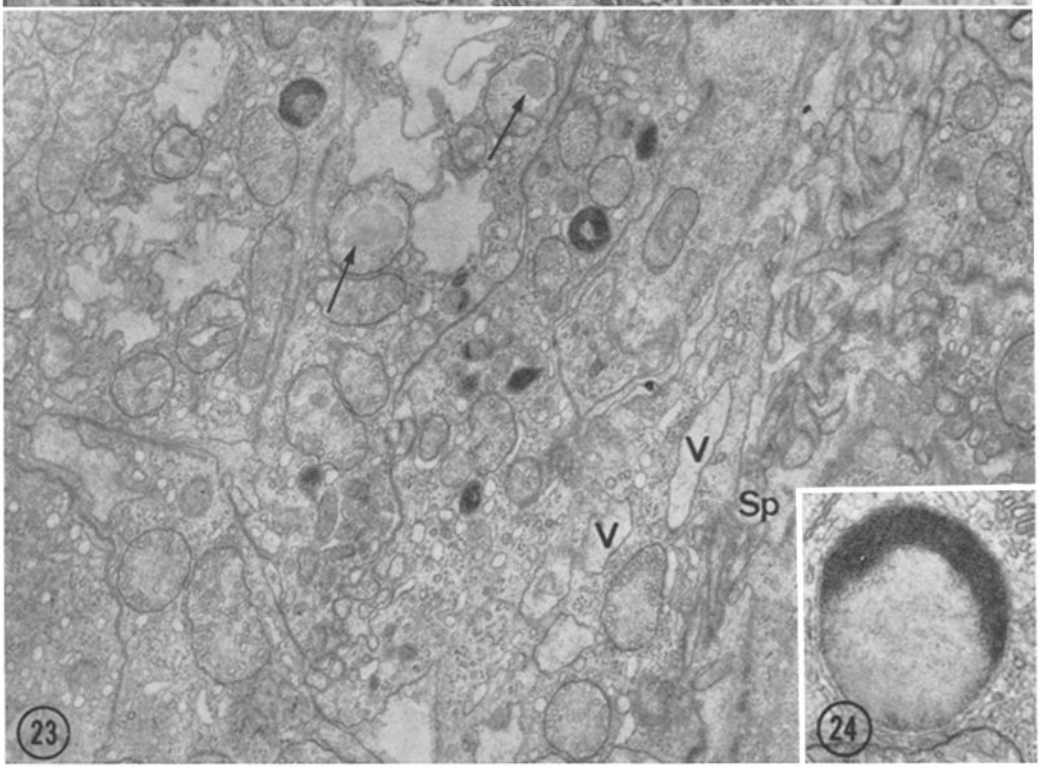
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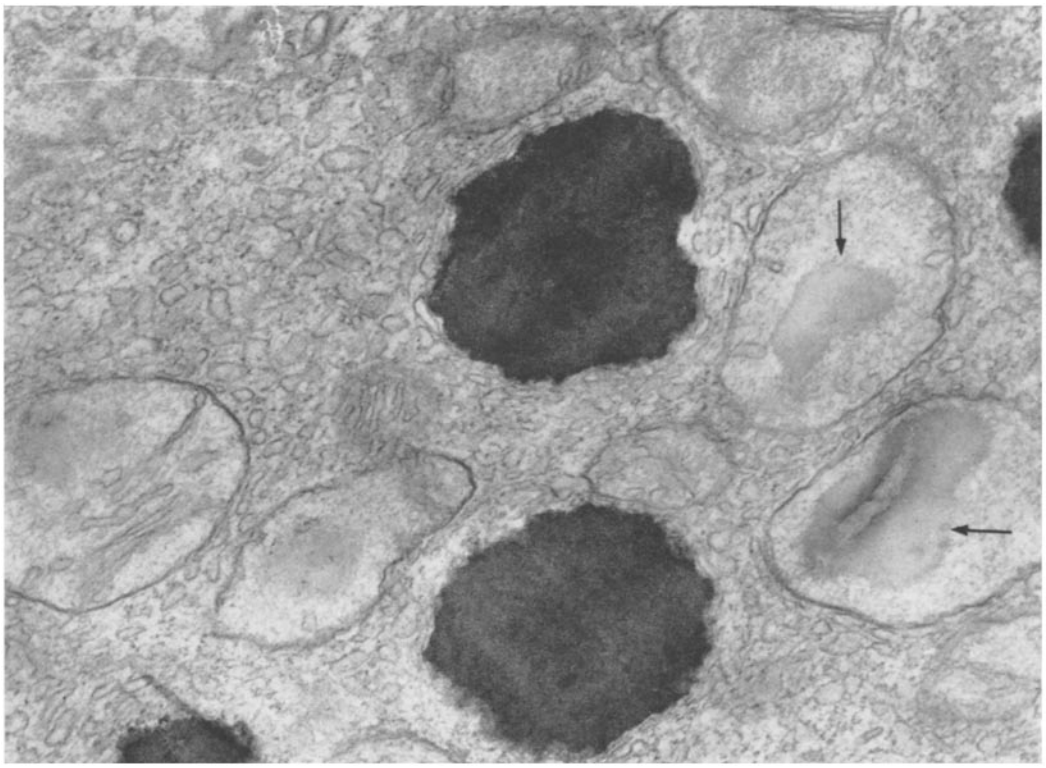

FIGURE 25 Several cytoplasmic inclusions are of greater electron opacity than the adjacent intramitochondrial deposits (arrows). 28 days of sodium deprivation. × 23,000.

FIGURE 22 Concentric membrane lamellae surround a portion of cytoplasm. Two dense bodies (arrows) are visible. 3 weeks of salt restriction. × 40,000.

FIGURE 23 Numerous dense bodies and intramitochondrial deposits (arrows). In addition, granular material is present in an intercellular space (*Sp*) which is similar to that seen in what appear to be intracellular vacuoles (*V*). 3 weeks of salt restriction. × 15,000.

FIGURE 24 One type of dense body which in this case consists of granular material of low electron opacity and parallel lamellae. 28 days of sodium deprivation. × 44,000.