OSMIUM STAINING OF ENDOPLASMIC RETICULUM AND MITOCHONDRIA IN THE RAT ADRENAL CORTEX

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ABSTRACT

The zona fasciculata of the rat adrenal cortex synthesizes and secretes glucocorticoids. As observed after aldehyde fixation, the cells in this zone contain an extensive endoplasmic reticulum (ER), a small Golgi apparatus, a moderate number of lipid droplets, and abundant mitochondria with tubulovesicular cristae. Numerous areas within the endoplasmic reticulum and mitochondrial cristae appear clear. In addition, a small percentage of mitochondria encompasses large, clear areas. After immersion of finely minced adrenal cortex in unbuffered 2% OsO₄ (40-48 hr at 40°C), deposits of osmium are seen within the Golgi apparatus, the entirety of the ER, and occasionally within mitochondria. In some mitochondria, the deposits are within cristae; in others, within vacuoles; in still others, in both cristae and vacuoles. These localizations correspond best to the clear areas found in aldehyde-fixed tissue. Osmium is not deposited in lipid droplets, in bar-containing inclusions, in mitochondrial matrix inclusions, or in the peripheral, outer mitochondrial spaces. Addition of zinc-iodide to OsO₄ increases the amount of Golgi apparatus and mitochondrial staining. Adrenocorticotropic hormone (ACTH) does not affect the localization of deposits; hypophysectomy decreases mitochondrial staining. This study (a) emphasizes the necessity for electron microscopic confirmation of osmium localization when this technique is used as a Golgi apparatus stain; and (b) suggests that the ER-staining pattern may be consistent in cells actively synthesizing steroids or steroid-like compounds.

INTRODUCTION

Immersion of mammalian tissues in unbuffered osmium tetroxide (OsO₄) for 40–48 hr frequently results in the deposition of a dense black precipitate in the outer, convex cisternae and smooth-surfaced vesicles of the Golgi apparatus (1–3). This pattern of deposition has proved useful as a cytochemical marker in electron microscopic studies of the epididymis, spermatozoa, intestine, pancreas, and central nervous system (1–6). In several cell types (1, 3, 5, 9), sparse to moderately heavy deposits of osmium-black have also been found within the nuclear envelope and cisternae of the endoplasmic reticulum.

This paper describes predominant extra-Golgi sites of OsO₄ staining in the endoplasmic reticulum and mitochondria of zona fasciculata cells of the rat adrenal cortex—cells particularly active in steroid synthesis and secretion. We also comment upon the appearance of these sites in paraformaldehyde-glutaraldehyde-fixed tissue, the effects of adrenocorticotropic hormone (ACTH) stimulation and hypophysectomy on the pattern of OsO₄ staining.
staining, and variations in the location and quantity of the deposits after adding zinc-iodide to OsO₄.

MATERIALS AND METHODS

Materials

Adrenal tissue was obtained from seven normal, two ACTH-stimulated, and three hypophysectomized mature male Sprague-Dawley rats, 44-196 days old. ACTH was administered to each animal in three subcutaneous injections of 0.1 ml (4 USP units) Acthar-Gel spaced over a 24 hr period before sacrifice. Hypophysectomized animals were obtained commercially (Simonson Laboratories, San Francisco, Calif.).


Fixation

The adrenal glands of ether-anesthetized animals were removed by ventral abdominal incision and minced into small blocks.

For morphologic examination, the tissue was fixed in 1% paraformaldehyde (10) and 3% distilled glutaraldehyde (11) in 0.1 M cacodylate buffer with 0.025 M CaCl₂ at a final pH of 7.4 for 4-5 hr at room temperature, followed by postfixation in 1% OsO₄ in Veronal-acetate buffer, pH 7.4, with 5% sucrose.

For osmium staining (3), the minced tissue was immediately placed in 2% OsO₄ in distilled, deionized water in vials wrapped with foil to exclude light. The solution was decanted and replaced by more of the same fixative after 24 hr; incubation was then continued for an additional 24 hr. The pH of the osmium solution decreased 3 pH units during this procedure.

To enhance osmium staining, a variation of the procedure was employed in several experiments. Metallic zinc-iodide was added to the OsO₄ according to the method of Niebauer et al. (12, 13). For this procedure, the tissue was immersed in the modified fixative at room temperature for 16-24 hr.

Subsequent Processing

After fixation, the tissue was processed in block with buffered 0.5% uranyl acetate with or without sucrose for 1 hr at room temperature. Rapid dehydration in an ascending series of ethanol was succeeded by immersion in propylene oxide and embedding in Epon. 1 micron sections were stained with toluidine blue for light microscopic examination. Silver-to-grey thin sections were then cut with a diamond knife on a Porter-Blum MT-2 microtome and mounted on Formvar- and carbon-coated copper grids. Sections were subsequently stained either with an aqueous solution of 3% uranyl acetate followed by Millonig's alkaline lead (14) or with Millonig's lead stain alone.

OBSERVATIONS

Normal Morphology (Aldehyde Fixation)

The cytoplasm of the large polyhedral cell of the zona fasciculata (Fig. 1) contains an abundant network of smooth-surfaced endoplasmic reticulum (better seen in Fig. 4), numerous spherical mitochondria with tubulovesicular cristae (Figs. 1 and 2), lipid droplets, short profiles of Golgi apparatus (Fig. 1, inset), and numerous clusters of ribosomes and glycogen. Although the fine structure of the zona fasciculata has already been described in detail elsewhere (15-20), characteristics of the two organelles particularly pertinent to this paper—mitochondria and endoplasmic reticulum—warrant additional comment.

MITOCHONDRIA: Mitochondria differ markedly in size, ranging from 2 to 5 µ in diameter. Densities in cristae and matrix also vary widely. Generally the matrix is fairly dense and contains abundant particles, 50-60 Å in diameter (Fig. 2), and rarely, 300-400-Å matrix granules. Occasionally, dense, amorphous masses (1-3 µ in diameter) similar to those described in corpora lutea (21) are found in the matrix (Fig. 8 A). A small percentage of mitochondria contains vacuoles from 0.4 to 4 µ in size, which are bound by a unit membrane and are less dense than identifiable cytoplasmic lipid droplets extracted during tissue preparation (Fig. 3). However, distinction between extracted lipid droplets and vacuoles is sometimes arbitrary.

Tubulovesicular cristae content also varies in density. Usually it is lighter than the mitochondrial matrix and apparently homogeneous. Occasional cristae appear clear (Fig. 2); however, such lack of density is uncommon in the peripheral, outer mitochondrial space. Occasionally, dense, lipid-like masses are visible within cristae of normal diameter as well as in some dilated cristae.
Abbreviations

a, amorphous mass
er, endoplasmic reticulum
G, Golgi apparatus
l, lipid
m, mitochondrion
n, nucleus
rer, rough-surfaced endoplasmic reticulum
ser, smooth-surfaced endoplasmic reticulum
v, vacuole

FIGURES 1-4 Paraformaldehyde-glutaraldehyde-fixed adrenocortical cells of normal rat zona fasciculata.

FIGURE 1 Low-magnification electronmicrograph illustrating the composition of an average cell in the zona fasciculata. l, lipid; m, mitochondrion; n, nucleus. Inset: Golgi apparatus (G). X 12,000; inset, X 36,000.
FIGURE 2. Large, spherical mitochondrion with tubulovesicular cristae. The contents of many cristae are of low density (arrows), comparable to that of ER (er). The mitochondrial matrix is dense and granular. \( \times 73,000 \).

tensive anastomotic network of endoplasmic reticulum is predominantly smooth-surfaced, with clusters of ribosomes near membranes but few attached to them. Flattened cisternae of the ER are closely apposed to the surfaces of lipid droplets and mitochondria (Figs. 2 and 4), while dilatations of the ER are noted throughout the cell. ER density, particularly in the dilated areas, is low and similar to that within mitochondrial cristae (Fig. 4). Some of the clear areas in both mitochondria and endoplasmic reticulum, of course, may be merely consequent to the plane of sectioning.

**OsO\(_4\) Staining**

OsO\(_4\) stains the endoplasmic reticulum in its entirety, including the nuclear envelope and the outer, convex cisternae and vesicles of the Golgi apparatus. In addition, mitochondrial vacuoles and cristae occasionally stain (Fig. 5). In general, mitochondrial staining is rare.

**Mitochondria** The staining spectrum in mitochondria is best illustrated by Figs. 6 A-F. In some of these, several central tubulovesicular cristae stain; in others, the majority of cristae; and in still others, cristae and central areas ap-
Figure 3 Variations in mitochondrial size, structure, and density. Medium-sized, small, and large mitochondrial vacuoles (v) are depicted in 3A, B, and C, respectively. Their low density is similar to that of lipid droplets (l) extracted during tissue preparation. 3A, X 15,000; 3B, X 45,000; 3C, X 46,000.

Deposits in cristae are invariably smooth and homogenous (Figs. 6 A–C), while those in vacuoles are coarsely granular (Figs. 6 C–F). Deposition does not occur in the outer mitochondrial space, despite a viable continuity with the intracristal space in many instances.
OsO₄ staining is not observed within dense, amorphous mitochondrial matrix (Fig. 8 B) and intracristal deposits or within lipid droplets surrounded by mitochondria (Fig. 9 B). Nor is it seen within the matrix granules.

Endoplasmic Reticulum and Golgi Apparatus Deposits within the cisternae of the ER and the nuclear envelope are nearly always coarse and granular, whereas deposits in the Golgi apparatus are generally homogeneous (Figs. 5, 7, and 11). No deposits are found within the coated vesicles of the inner, concave surface of the Golgi apparatus.

Except for the ER, Golgi apparatus, and mitochondria, no osmium-black is deposited within other organelles or inclusions, neither in the bar-containing bodies (Fig. 10 B) reported to be composed of cholesterol (22) nor in the lipid droplets (Figs. 5, 7, 9 B, and 11), which have a high content of free cholesterol (23). Moreover, osmium-black is not generally found in extracellular or vascular spaces (Figs. 5, 11, and 12).

ACTH Stimulation and Hypophysectomy

As other investigators have noted, ACTH stimulation causes hypertrophy of the zona fasciculata cells in general and the Golgi apparatus in particular (24, 25). In addition (25), we also observe a greater number of large and small coated vesicles. ACTH has no notable effect on OsO₄ staining in the endoplasmic reticulum or mitochondria, but frequently staining is increased in Golgi cisternae and smooth vesicles (Fig. 11, inset). The capriciousness of this reaction is evident in Fig. 11, which

![Figure 4](image-url) Dilated areas of the ER are of lower density (arrows) than that of the lipid droplet (I) surrounded by flattened ER (er) cisternae depicted here. The stippling due to peripheral fibers of this type of lipid inclusion may be analogous to such mottling in lipid droplets found within adipose cells (39). × 61,000.

Figure 4

DANIEL S. FRIEND AND GAIL E. BRASSIL OsO₄ Staining in ER and Mitochondria
Cells of the normal rat zona fasciculata, prepared by immersion in unbuffered 2% OsO₄ for 40–48 hr.

**Figure 5** OsO₄ staining of the ER, Golgi apparatus (G) (inset), and mitochondrion (arrow). Lipid (l) is retained but does not stain. × 6000; inset, × 33,000.

Atrophy of the zona fasciculata cells, particularly evident as diminished endoplasmic reticulum and Golgi apparatus, is induced by hypophysectomy. Most mitochondrial cristae become lamellar in configuration. OsO₄ staining persists in the atrophic ER and Golgi apparatus, but is absent in mitochondria (Fig. 12).

**OsO₄-Zinc-Iodide**

The addition of zinc-iodide to OsO₄ impairs good preservation of the tissue but markedly increases the number of mitochondrial cristae which...
FIGURE 6 Smooth, homogeneous OsO4-staining of mitochondrial cristae (6 A–C) and coarse, granular staining of mitochondrial vacuoles and ER (6 C–F). The sequence depicted (A–F) represents our interpretation of OsO4-positive droplet formation in mitochondria. Arrow, mitochondrial membranes. 6 A, × 38,000; 6 B, × 86,000; 6 C, × 33,000; 6 D, × 43,000; 6 E, × 37,000; 6 F, × 12,000.
stain in normal animals (Fig. 13), resulting in deposits within most mitochondrial cristae in a high percentage of cells. The majority of saccules and vesicles of the Golgi apparatus, as well as many residual bodies, including lipofuscin pigment, also stain heavily.

DISCUSSION

Topographical Localization

This study presents a pattern of OSO4 staining notably different from the staining pattern of 260

FIGURE 7 OsO4-positive droplet, presumably formed within a mitochondrion, is limited by unit membranes (arrow) not resolved at this magnification. Neutral lipid (l) droplets do not stain and are not limited by unit membranes. er, endoplasmic reticulum. X 24,000.

saccules and vesicles on the outer, convex surface of the Golgi apparatus reported in other tissues (1-3). In addition to staining the Golgi apparatus of the adrenal cortex, OsO4 heavily stains both the rough- and smooth-surfaced endoplasmic reticulum and some mitochondria. This pronounced staining is significant for several reasons: (a) It emphasizes the necessity for careful ultrastructural confirmation of OsO4 staining sites when this technique is used as a Golgi marker for light microscopic studies—and especially the need for confirming the results of early studies involving the

FIGURES 8 A, 9 A, and 10 A Micrographs of tissue fixed in paraformaldehyde-glutaraldehyde. Figures 8 B, 9 B, and 10 B Similar fields from tissue immersed in unbuffered OsO4 for 40-48 hr.

FIGURE 8 Amorphous mitochondrial mass (a) seen in aldehyde-fixed tissue (8 A) does not stain with OsO4 (8 B). 8 A, X 39,000; 8 B, X 36,000.

FIGURE 9 Lipid (l) droplet encircled by cup-shaped mitochondrion (m) (9 A) does not stain with OsO4. G, Golgi apparatus. 9 A, X 26,000; 9 B, X 18,000.

FIGURE 10 Bar-shaped cholesterol inclusion (arrow) is extracted to the same degree as the contents of mitochondrial vacuoles (v) in aldehyde-fixed tissue (10 A). Immersion in OsO4 preserves but does not stain the inclusion (arrow). 10 A, X 32,000; 10 B, X 36,000.

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FIGURE 11 ACTH stimulation usually increases staining in the hypertrophic Golgi apparatus (G) (inset), but the capricious nature of the reaction is evident in the paucity of Golgi staining in the main figure of another ACTH-stimulated cell. × 12,000; inset, × 27,000.
FIGURE 12  Hypophysectomy decreases staining within mitochondria and the atrophic ER (er). Note lamellar-type cristae in mitochondria (m) in the inactive zona fasciculata. × 17,000.

central nervous system wherein this technique has been widely employed (4, 5). (b) Our findings suggest that ER staining may represent a consistent pattern in cells where the intermediates of cholesterol and steroid biogenesis are present in large concentration. Highly concentrated, non-saponifiable lipids, for example, are found in the zona fasciculata cells concerned with corticosterone synthesis, in corpora lutea involved in progesterone synthesis, in interstitial cells of the testis active in testosterone synthesis, in hepatocytes participating in bile acid synthesis, and in intestinal absorptive epithelial cells implicated in cholesterol biosynthesis (25). Intermediates in the biosynthetic pathways of these secretory products are very similar and the ER staining pattern is consistent in all the tissues mentioned (unpublished observations). If this ER staining pattern proves constant for all cells active in synthesizing steroids or steroid-like compounds, the technique will then become useful for identifying cell types active in steroidogenesis in heterogeneous tissues such as the epididymis. Thus far, only biochemical or physiologic evidence of such activity is available (27, 28). (c) Since mitochondrial-vacuole staining is uniform, even in vacuoles with diameters exceeding the section thickness, our results indicate that OsO₄ staining can occur within the content of a compartment as well as in close association with membranes. Frequently, as in Golgi-apparatus staining, the chemical groups, double bonds, and polar ends of lipid molecules responsible for cyclic monoester and OsO₄ formation appear to be membrane constituents. But in mitochondrial vacuoles, the substances responsible for OsO₄ staining clearly appear to be in the vacuolar content. (d) The areas of osmium-black localization best correspond to the clear areas seen in paraformaldehyde-glutaraldehyde-fixed tissues—namely, ER dilatations, mitochondrial vacuoles, and some
In the normal animal, cristae of most mitochondria (m) stain with zinc-iodide added to OsO₄. However, cells fix poorly with this mixture (compare with Fig. 6), as indicated by shrinkage of mitochondrial cristae and distortion of the endoplasmic reticulum. Moreover, the Zn-I-OsO₄ precipitate is finer than that following conventional osmium staining. The specificity of this reaction and the degree of analogy with OsO₄ staining are uncertain. 1, lipid droplet. X 20,000.

Mitochondrial cristae. Steroids are easily extracted during dehydration after aldehyde fixation (20, 23, 28, 29), but it is likely that OsO₄ fixation retains such compounds (30), and prolonged immersion may stain them. Osmium-black favors the clear areas, either indicating the possible presence of steroids and a specific reaction there, or suggesting that the material requires suitable spaces for reduction and precipitation. As observed in the adrenal cortex, steroids may be at least partially responsible for the staining reaction and pattern, whereas cholesterol and neutral lipids are not. The nature of any possible relationship between intermediates or cofactors in steroid biogenesis and OsO₄ staining is unknown; however, the localization of deposits in all compartments directly involved in the production of corticosterone in the adrenal gland, and the fact that staining of these compartments decreases with reduced hormone production in hypophysectomized animals, imply that a relationship does indeed exist.

**Zinc-Iodide-OsO₄ Staining**

In view of the observation that the vesicles of cholinergic nerve endings stain with Zn-I-OsO₄ mixtures, several investigators have assumed this procedure to be specific for localizing acetylcholine (31, 32). Contrary to this assumption, our findings indicate that this reaction is not selective to that degree. The mixture also stains Golgi vesicles and sacculles, endoplasmic reticulum, mitochondria, and secondary lysosomes. It is probable, as in OsO₄ staining, that reactive chemical groups (33–38) of fairly ubiquitous compounds become available for interaction with the staining mixture during the procedure.

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