CYTOCHEMICAL STAINING OF MULTIVESICULAR BODY AND GOLGI VESICLES

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ABSTRACT

To investigate the origin and nature of vesicles found within multivesicular bodies (mvb), the cytochemical staining properties of mvb vesicles were compared with those of other cytoplasmic vesicles, i.e. those associated with the Golgi complex and endocytic vesicles found near the apical cell surface. Rat epididymal tissue was stained in unbuffered OsO_4 for 40–48 hr, and the distribution of stain was compared to that of reaction products for acid phosphatase (AcPase) to mark lysosomal vesicles, or thiamine pyrophosphatase (TPPase) to mark certain Golgi vesicles, or infused with peroxidase (HRPase) to demonstrate endocytic vesicles. Mvb vesicles were stained only by OsO_4; AcPase, TPPase, and HRPase reaction products stained the mvb matrix. OsO_4 also stained certain vesicles along the convex surface of the Golgi complex. The findings suggest that mvb vesicles in epididymal epithelium are not lysosomes and are not involved in protein uptake. The majority of these vesicles have cytochemical reactions in common with vesicles located along the convex surface of the Golgi complex and may be derived therefrom. A minority are derived from the mvb-limiting membrane.

INTRODUCTION

The term multivesicular body (mvb) is commonly used to describe a vacuole containing small vesicles. These organelles are numerous in principal cells of the rat vas deferens and cauda epididymis, in which, as in many other epithelia, they function as digestive vacuoles or phagolysosomes (1). The origin of the vesicles within mvb, as well as the functional relationship between mvb vesicles and other groups of vesicles in the cell, have remained elusive. Previous work on the vas deferens (2) has shown that the morphologic and cytochemical properties of mvb vesicles are different from those of endocytic vesicles transporting exogenous protein from the cell surface or of vesicles transporting hydrolytic enzymes from the Golgi region to heterolysosomes.

In the present study, OsO_4 staining (3, 4) was used to study the origin and nature of mvb vesicles in the epididymis. The distribution of the OsO_4 stain was compared to that of other cytochemical markers. It was found that mvb vesicles have cytochemical reactions in common with one particular group of vesicles found in the Golgi region, the function of which is unknown. No evidence was obtained to support the widely reported view that vesicles within the mvb contain hydrolytic enzymes and represent primary lysosomes.

MATERIALS AND METHODS

Materials

Observations were made on the lining epithelium of the cauda epididymis from six normal, 10-wk-old, male Sprague-Dawley rats and from three rats

hypophysectomized[1] 17–21 days before sacrifice. Hypophysectomized rats were purchased from Simonsen Laboratories, Gilroy, California.

The following reagents and substrates were obtained from Sigma Chemical Co., St. Louis, Mo.: β-glycerophosphate (Type I); cytidine 5′-monophosphate (disodium salt); 3,3′-diaminobenzidine tetrahydrochloride; horseradish peroxidase (Type II); and thiamine pyrophosphate (cocarboxylase). Osmium tetroxide was obtained from Merck & Co., Inc., Rahway, N.J., and Goldsmith Division of National Laboratories, Chicago, Ill.

Methods

Morphologic Studies

Small blocks of epididymis were fixed at 4°C for 2 hr in acetate-Veronal-buffered 1% OsO_4 (pH 7.4) with 5% added sucrose. After fixation, the tissue was treated at room temperature for 1½ hr with buffered 0.5% uranyl acetate (5) containing 4% sucrose, quickly dehydrated in graded ethanols, and embedded in Epon 812 (6). Some specimens were dehydrated directly after fixation.

Thin sections (50 mμ) were prepared with a Sorvall MT-2 microtome (Sorvall, Ivan, Inc., Norwalk, Conn.) equipped with a diamond knife and were stained with alkaline lead (7) or 5% aqueous uranyl acetate and lead.

Electron micrographs were taken at original magnifications of 3,000–30,000 with a Siemens Elmiskop I or Ia operating at 80 kv with a double condenser and a 50 μ objective aperture.

Cytochemical Studies

OSO4-STAINING: The epididymis was cut into small pieces, immersed in unbuffered 2% OsO_4 (pH 6.2–6.8) in foil-wrapped vials, and placed in an oven at 40°C (4). After 24 hr, the tissue was drained, fresh 2% OsO_4 was added, and the tissue was returned to the oven for another 16–24 hr. The tissue was then drained, treated for 1½ hr with 0.5% uranyl acetate, dehydrated in graded ethanols, and embedded in Epon.

PEROXIDASE INFUSION: 6% horseradish peroxidase (8) in isotonic saline was slowly injected into the vas deferens of lightly anaesthetized animals (2). Retrograde flow from the site of injection filled the distal epididymal tubules with the tracer enzyme.

FIXATION FOR ENZYME CYTOCHEMISTRY: The cauda epididymis was fixed in cold 1% paraformaldehyde (9) and 3% distilled (5) glutaraldehyde, in 0.067 M sodium cacodylate buffer, pH 7.4. After fixation, the tissue was washed overnight in 0.05 M acetate-Veronal buffer, pH 7.4, with 7% sucrose. Nonfrozen sections were prepared (10) on a Smith-Farquhar TC-2 tissue sectioner (Sorvall, Ivan, Inc., Norwalk, Conn.) and incubated in the following substrates:

Peroxidase. Tissue fixed for 3–4 hr was incubated at 20°C for 30 min at pH 7.6 in the diaminobenzidine medium of Graham and Karnovsky (8). Controls were incubated in media without diaminobenzidine or without peroxidase.

Acid Phosphatase. Tissue fixed for 2 hr was incubated at 37°C for 1½ hr (2) in media prepared with β-glycerophosphate as substrate according to Barka and Anderson (11), or with cytidine monophosphate at pH 5.0 according to Novikoff (12). Controls were incubated either with 0.1 M NaF added or with the substrate omitted.

Thiamine Pyrophosphatase. Tissue fixed for 3 hr was incubated for 1½ hr at pH 7.4 in the medium employed by Novikoff and Goldfischer (13). Controls were incubated at pH 5.0 or with 0.1 M NaF added to the medium (pH 7.4), or without the substrate (pH 7.4).

Subsequent Processing of Tissue. The tissue was postfixed for 1½ hr at room temperature in acetate-Veronal-buffered 1% OsO_4 (pH 7.4) with 5% sucrose, treated with buffered 0.5 M uranyl acetate for 1 hr at room temperature, dehydrated in graded ethanols, and embedded in Epon. Thin sections were prepared in the same manner described for morphologic studies.

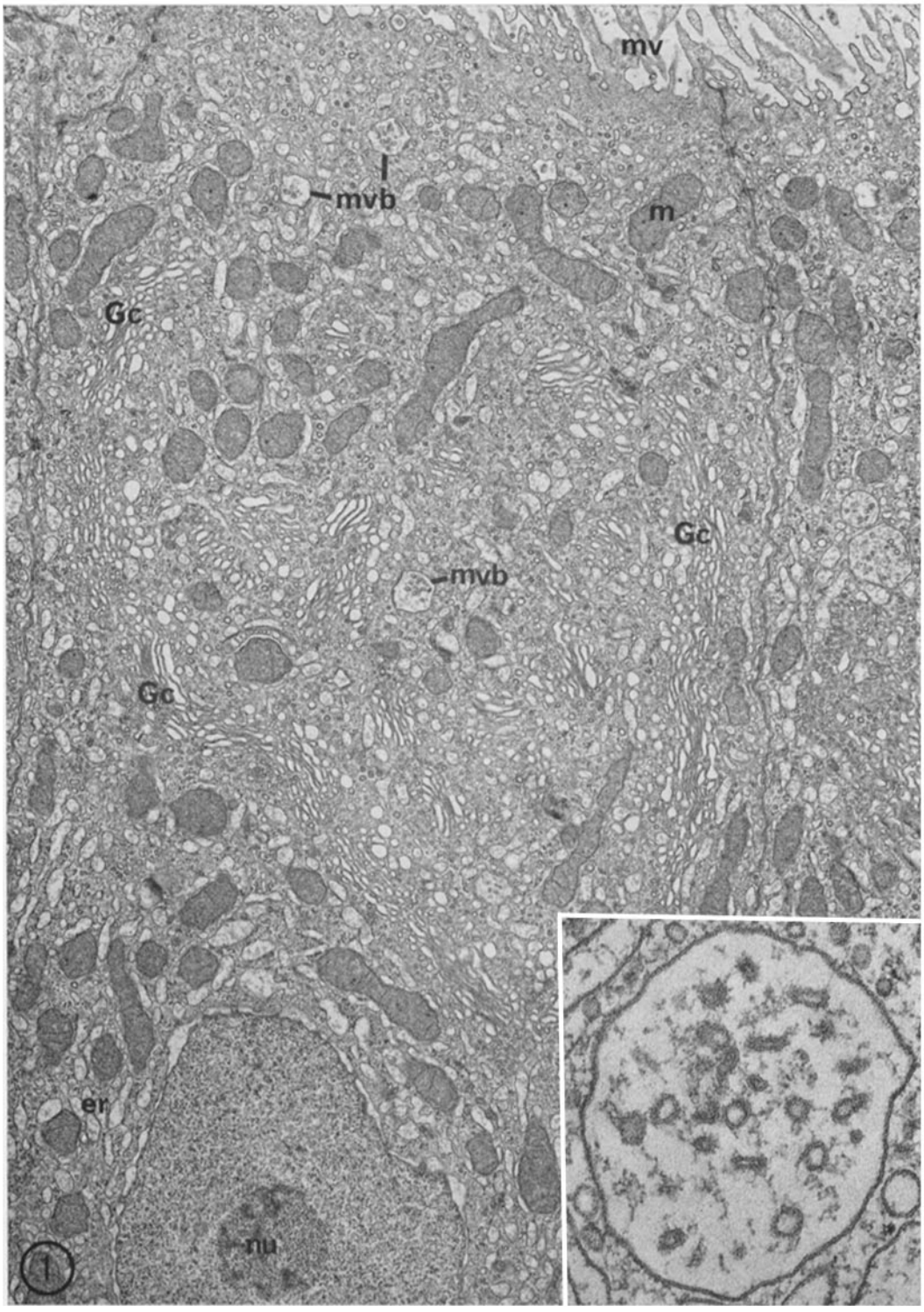
OBSERVATIONS

Morphology

The structure of the principal cell of the normal rat cauda epididymis is similar to that described previously (14), for the cauda epididymis in other species (15, 16), and for the rat vas deferens (2, 17). A section from the normal rat cauda epididymis is shown in Fig. 1. Hypophysectomy caused a decrease in the height of the epithelial cell but did not alter its fine structural topography (Fig. 2). The organelles of interest, namely the mvb, Golgi complex, and various smooth-surfaced vesicles, will therefore be described together for both the normal and hypophysectomized animals.

MULTIVESICULAR BODIES: Multivesicular bodies are found singly or in clusters in the apical region of the cell. The majority of the bodies are large (500 mμ in diameter). They have a thick (10 mμ) limiting membrane, a matrix of low density, and sparse smooth-surface vesicles (20–80 mμ in diameter), the membranes of which are also about 10 mμ in diameter (Fig. 1, inset). Frequently, a clump of finely particulate material is

[1] Hypophysectomy increases OsO_4-staining of mvb and outer Golgi vesicles.



d, dense mass
er, endoplasmic reticulum
Gc, Golgi complex
m, mitochondrion
mv, microvilli
mvb, multivesicular body
nu, nucleolus
v, small vesicles
V, large vesicles

FIGURE 1 Columnar epithelial cell from the cauda epididymis. The lower third of the cell is occupied by short segments of rough-surfaced endoplasmic reticulum (*er*) and by an elongate nucleus with a prominent nucleolus (*nu*). The upper two-thirds of the cell contains an extensive horseshoe-shaped Golgi complex (Gc). An outer, convex surface and an inner, concave one are apparent in most sections through the Golgi stack. Multivesicular bodies (*mvb*) and a multitude of small vesicles are scattered throughout this large Golgi region. Mitochondria (*m*) are moderately numerous. Microvilli (*mv*). × 14,000. *Inset:* Large mvbs such as this one with a few vesicles and a pale matrix are common in the apical cytoplasm. × 82,000.

FIGURE 2 This low-magnification picture of the rat epididymis illustrates extensive OsO_4-staining of Golgi cisternae and vesicles, clusters of vesicles in the apical cytoplasm (arrows), and vesicles within mvbs. × 9,000.

present in the matrix (Fig. 6). Nicander (18) refers to these large vacuoles with few vesicles and a light matrix as light mvb. The mvb in the sections shown in Figs. 1 and 6 correspond to the light or Type I (19) mbv. A dark mbv, as shown in Fig. 5, is smaller (200–400 mμ in diameter) than the light one and has a dense, uniformly granular matrix and numerous smooth-surfaced vesicles. In general the dark type of mvb is less numerous than the light one and is found in close apposition to stacks of Golgi cisternae.

GOLGI CISTERNAE AND VESICLES: The Golgi complex forms an extensive supranuclear collar consisting of an extensive stack of parallel, smooth-surface cisternae and both smooth-surfaced and coated (20, 2) vesicles (Figs. 1–4, 7–9, 12). In most sections through the stack of Golgi cisternae, an inner, concave surface and an outer, convex surface are apparent (2, 6) (Figs. 1, 4, 7–9). Clusters of smooth-surfaced vesicles (40–80 mμ in diameter) are found associated with cisternae of the outer, convex Golgi surface (Fig. 4) at the ends of the cisternal stacks (Figs. 2 and 3), adjacent to transitional elements of the rough ER (Fig. 13). Transitional elements are the partly rough- and partly smooth-surfaced cisternae of the ER found

near the Golgi complex. Smooth-surfaced vesicles are also found in smaller numbers along the concave Golgi surface. Coated vesicles (750 mμ in diameter) are interspersed with the smooth-surfaced vesicles at the ends of Golgi cisternae and along the concave surface of the membranous stacks.

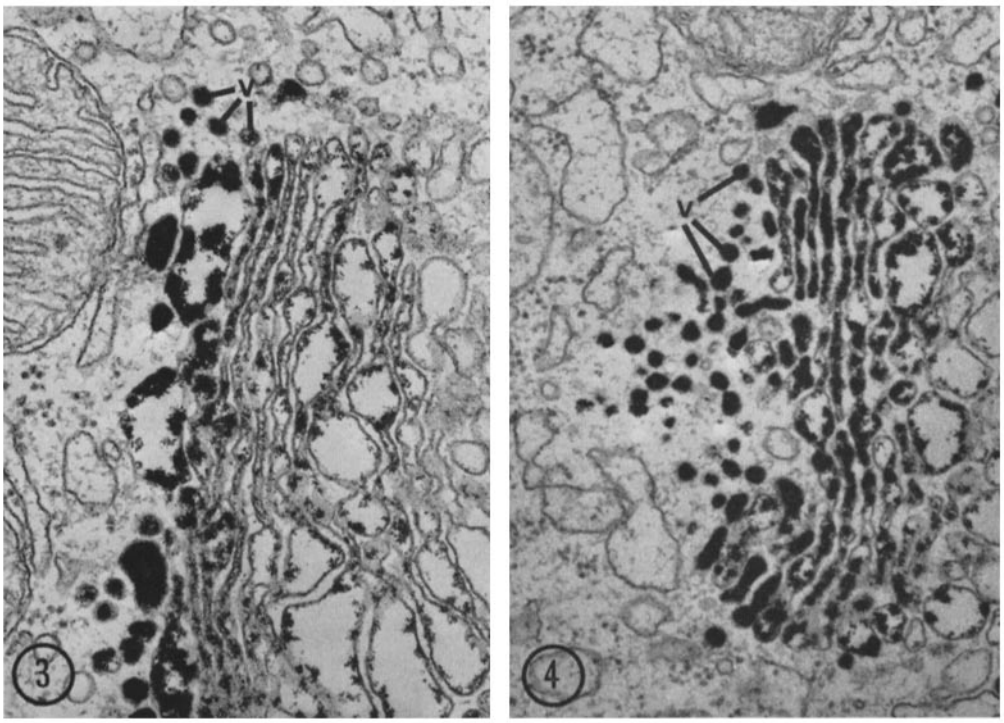
OsO_4 Staining

MVB AND GOLGI VESICLES: In the normal rat epididymis OsO_4 intensely stained (*a*) some smooth vesicles within mvb (Figs. 15, 16), (*b*) smooth vesicles along the outer, convex surface of the Golgi complex (Fig. 3), (*c*) some smooth vesicles adjacent to transitional elements of the rough ER, and (*d*) 2–3 outer Golgi cisternae (Figs. 2, 7, and 8).

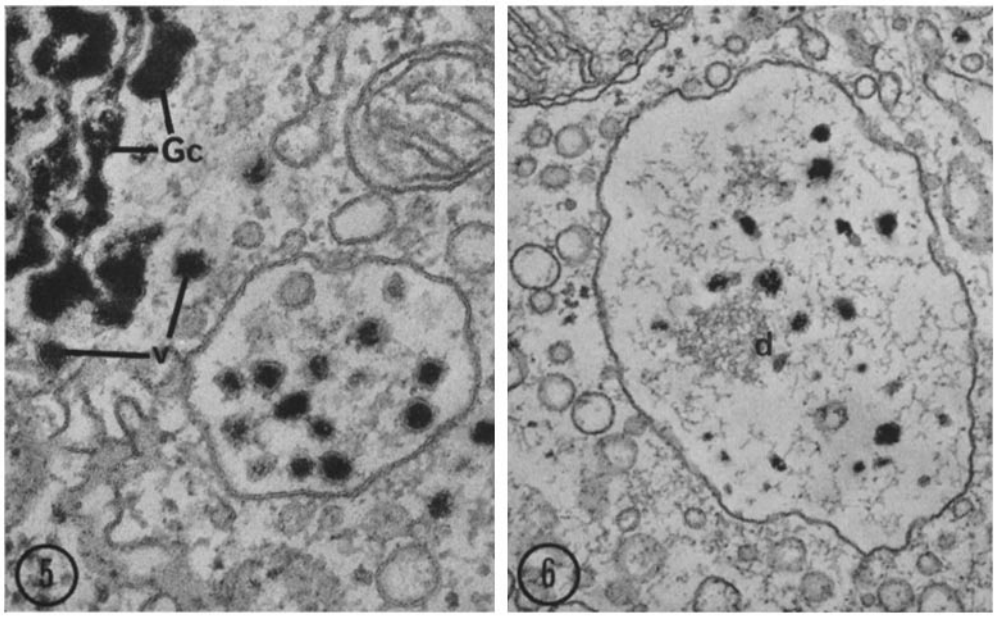
In the hypophysectomized rat, the sites of intense OsO_4-staining were the same as in the normal rat, but at each site a larger number of organelles contained the OsO_4 reaction product. Most of the smooth vesicles within both light and dark types of mvb (Figs. 2, 5, and 6), many more smooth vesicles at the ends of all Golgi cisternae (Figs. 2 and 4), and most of the smooth-surfaced vesicles adjacent to transitional elements of the rough ER contained heavy deposits (Figs. 2 and 13).

In both normal and hypophysectomized animals OsO_4 did not stain large or small coated vesicles (the coats of which survive prolonged osmication well) the mvb matrix, or the majority of smooth vesicles along the inner, concave surface of the Golgi complex.

ENDOPLASMIC RETICULUM: Smooth- and rough-surfaced ER were stained in a small percentage of the cells (Figs. 7 and 8). The ER stained less intensely than did elements of the Golgi complex. This difference in staining intensity was so consistent that it could be used as a criterion for distinguishing cisternae of the two organelles. In rare instances, the cisternae which



FIGURES 3 and 4 Golgi complexes from normal and hypophysectomized animals. In the normal rat epididymis (Fig. 3), OsO_4 stains the outer, convex cisternae and vesicles (*v*) intensely and occasionally stains the intermediate and inner Golgi cisternae lightly, in the same manner that it stains the ER in a small percentage of cells. Note that the sites of staining are the same in the hypophysectomized rat (Fig. 4), but that the amount of stain is greater than in the normal one. Fig. 3 × 50,000. Fig. 4 × 42,000.



FIGURES 5 and 6 The epididymis has small, dark mvbs in the central Golgi region (Fig. 5) and large, light mvbs in the apical cytoplasm (Fig. 6). OsO_4 stains vesicles within both types of mvb, as well as the Golgi cisternae (Gc) and vesicles (*v*). A fibrillar dense area (*d*) is commonly seen in large, light mvbs. Fig. 5 × 72,000. Fig. 6 × 45,000.

stained with the same intensity as ER, and therefore were considered continuous with it, lay along the inner, concave surface of the Golgi complex (Figs. 7 and 8). They had the intercisternal spacing normally seen between Golgi cisternae (Fig. 8). Inasmuch as they appeared continuous with the ER, had the intercisternal spacing characteristic of the Golgi complex, and were AcPase-positive, they correspond to GERL (21, 22) described in other tissues.

Smooth-surfaced cisternae of indeterminate origin occasionally stained intensely with OsO_4. They encircled and segregated small areas of the cytoplasm in the Golgi region which included segments of ER, vesicles, and cytoplasmic matrix (Fig. 14), and are thus, by definition, early forms of autophagic vacuoles (1).

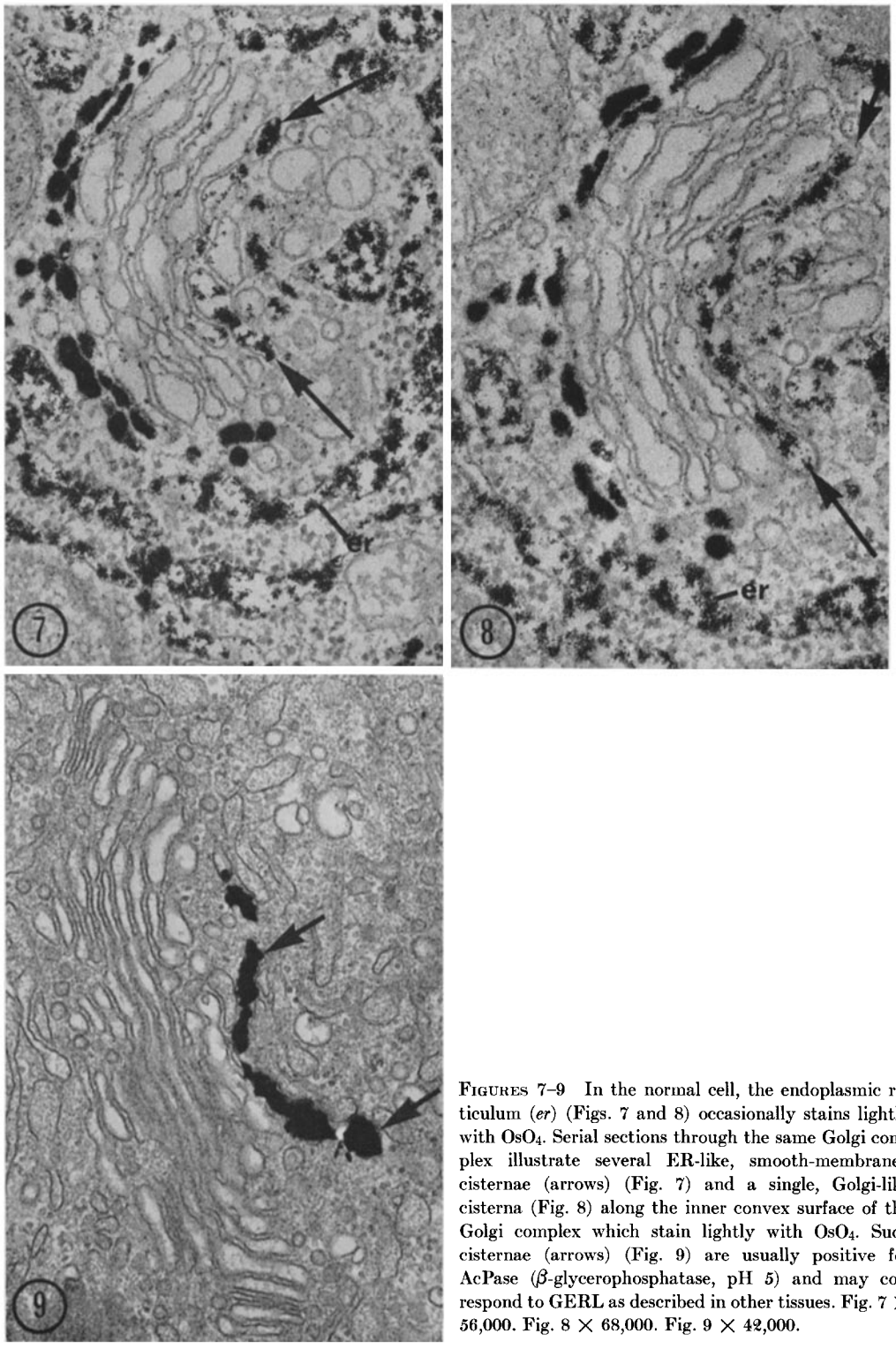
Enzyme Cytochemistry

The topographic localization of HRPase, AcPase, and TPPase reaction products was the same in both the normal and hypophysectomized rats and the same as described previously for the vas deferens (2). In contrast to the localization of OsO_4-staining within mvb vesicles, the reaction products of HRPase, AcPase, and TPPase were confined to the mvb matrix.

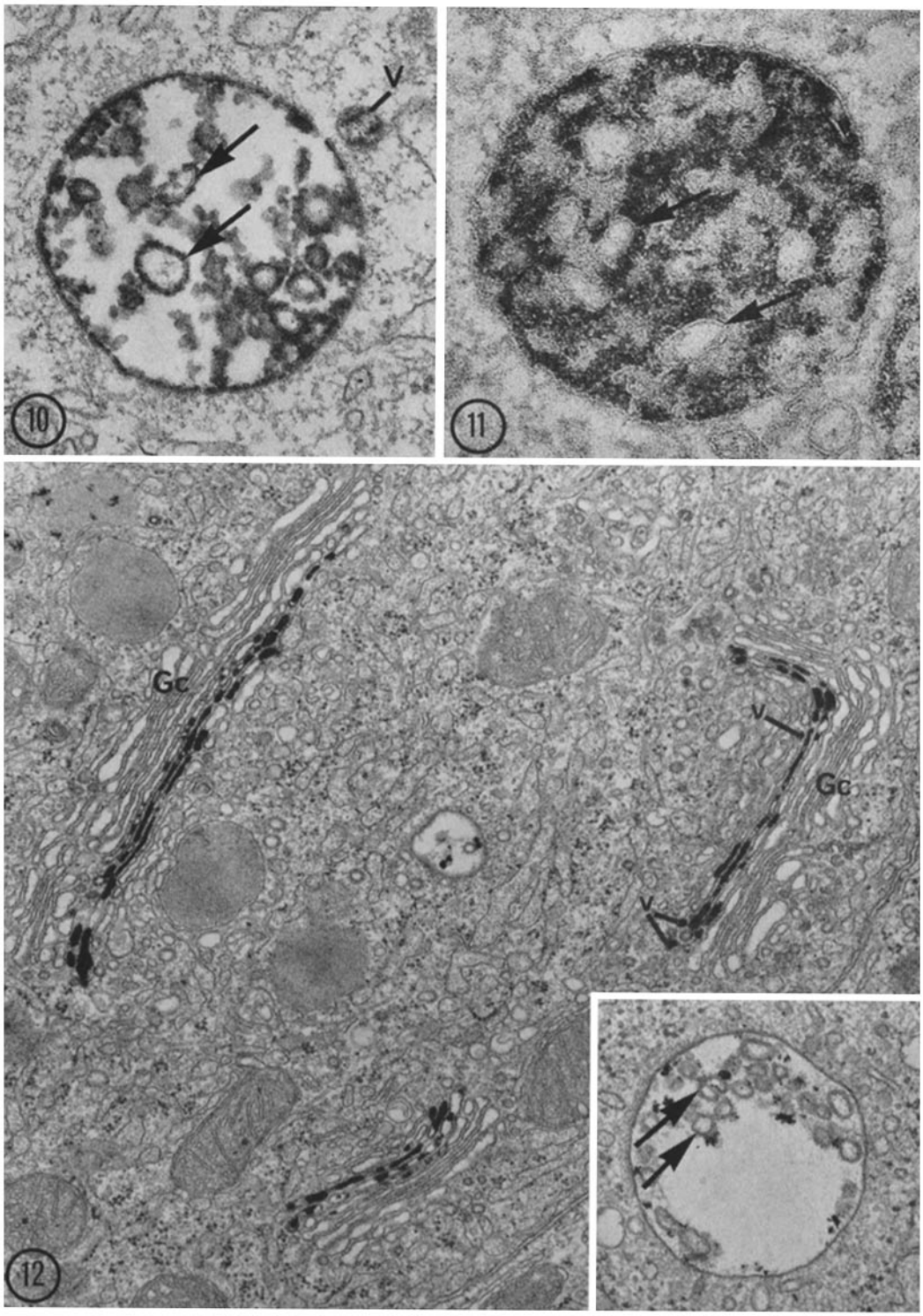
HORSERADISH PEROXIDASE (HRPase): 40 min after the infusion of HRPase into the lumen of the cauda epididymis, HRPase was localized in: coated intermicrovillar pits, large coated and smooth-surfaced vesicles in the apical cytoplasm, and the matrix of the mvb (Fig. 10).

ACID PHOSPHATASE (ACPase): Reaction product was found in decreasing concentration in: dense bodies, autophagic vacuoles, mvb, and the inner Golgi cisterna (Fig. 9) and associated smaller vesicles. Within the mvb, the reaction product was confined to the matrix (Fig. 11). The product was not found in any other populations of vesicles, i.e. those associated with the outer Golgi cisternae, those adjacent to transitional elements of the rough ER, or those located near the apical cell surface.

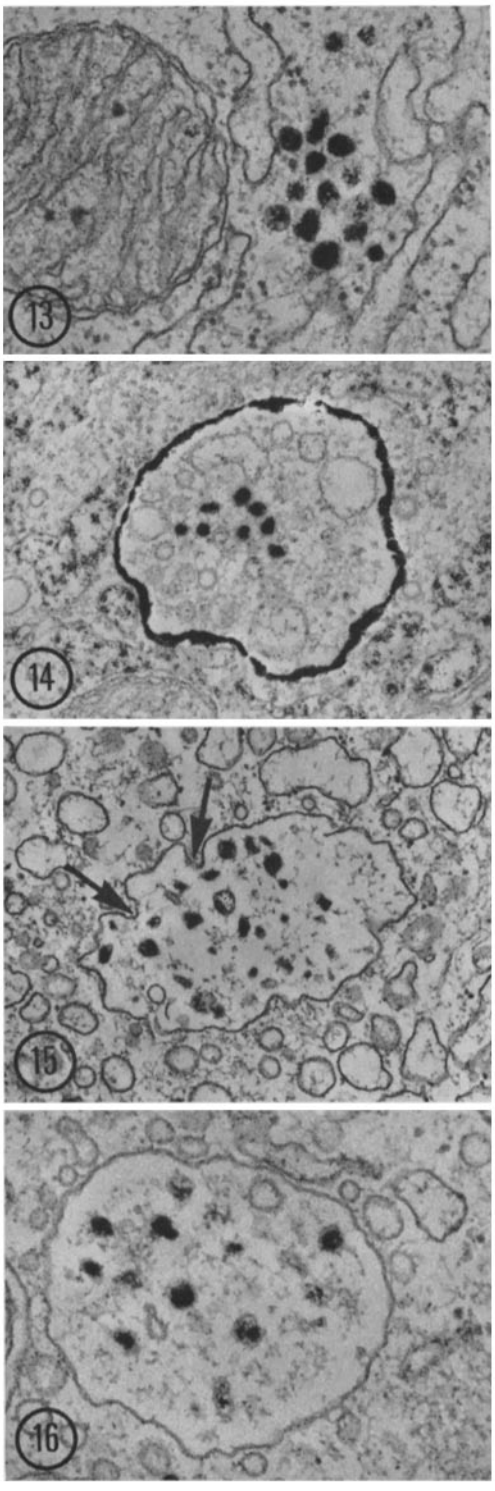
THIAMINE PYROPHOSPHATASE (TPPase): Reaction product was consistently found in 2–3 cisternae along the concave surface of the Golgi complex and in the small vesicles associated with these cisternae (Fig. 12). The inner Golgi cisterna was sometimes negative for TPPase; the adjacent



FIGURES 7–9 In the normal cell, the endoplasmic reticulum (*er*) (Figs. 7 and 8) occasionally stains lightly with OsO_4. Serial sections through the same Golgi complex illustrate several ER-like, smooth-membraned cisternae (arrows) (Fig. 7) and a single, Golgi-like cisterna (Fig. 8) along the inner convex surface of the Golgi complex which stain lightly with OsO_4. Such cisternae (arrows) (Fig. 9) are usually positive for AcPase (β-glycerophosphatase, pH 5) and may correspond to GERL as described in other tissues. Fig. 7 × 56,000. Fig. 8 × 68,000. Fig. 9 × 42,000.



FIGURES 10–12 These figures illustrate sites of distribution of other cytochemical markers (HRPase, AcPase, TTPase) to contrast with sites of OsO_4-staining. Note in all these cases that reaction product is confined to the matrix of mvbs and is not seen within mvb vesicles (arrows). In Fig. 10, HRPase is present in a large vesicle (*V*) and in the matrix of an mvb 40 min after its introduction into the lumen of the epididymis. In Fig. 11, AcPase (cytidine 5-monophosphate, pH 5) reaction product is found exclusively in the matrix of those mvb which are reactive with this procedure. In Fig. 12, TPPase reaction product is found in cisternae and vesicles (*v*) along the inner, concave surface of the Golgi complex (*Gc*) and is also seen rarely in the matrix of an mvb (inset). Fig. 10 × 78,000. Fig. 11 × 130,000. Fig. 12 × 25,000. Inset × 45,000.



2–3 Golgi cisternae were always strongly reactive. Scant reaction product was occasionally found in the mvb matrix; it was never present in the mvb vesicles (Fig. 12, inset).

DISCUSSION

Nature and Origin of mvb Vesicles

The findings in this study reveal that vesicles within the dark and light types of mvb of epididymal cells are similar in their morphologic characteristics and cytochemical staining properties to outer Golgi vesicles, i.e. those located adjacent to transitional elements of the rough ER, at the ends of Golgi cisternae, or along the outer, convex surface of the Golgi complex, since all these vesicles stain with OsO_4 but are not stained by the other cytochemical procedures used (AcPase, TPPase, HRPase). It is probable that the mvb and outer Golgi vesicles share a common origin, but in the epididymis it is not known whether that origin is from Golgi or ER elements or from both. Their lack of staining with AcPase and HRPase in our findings make it unlikely that they are endocytic or lysosomal.

As in other tissues (2, 23–26), tracers transported from the cell surface to the mvb appear within the mvb matrix. Since mvb vesicles did not contain HRPase, which marks endocytic vesicles, they do not appear to be endocytic in origin.

Several investigators at one time regarded vesicles within the mvb as primary lysosomes (27). The present observations, as well as those reported previously (2, 9), do not support this view, since AcPase reaction product was confined to the mvb matrix and was not seen within the vesicles. Moreover, in the epididymis, the sites of

FIGURES 13–16 illustrate how an mvb may form through the mechanism of autophagy. Clusters of OsO_4-stained smooth vesicles (Fig. 13) are occasionally encircled by an OsO_4-staining cisterna of indeterminate origin (Fig. 14). "Compaction" of the membranes with the loss of OsO_4-staining content of this cisterna and the budding of vesicles from the mvb membrane invaginations (arrows) (Fig. 15) would result in the formation of an mvb like that depicted in Fig. 16. In this mvb, most of the vesicles are stained with OsO_4, but two are not. Scattered densities in the mvb matrix are comparable in appearance to those in the cytoplasmic ground substance. Fig. 13 × 48,000. Fig. 14 × 44,000. Fig. 15 × 30,000. Fig. 16 × 76,000.

AcPase reaction product and intense staining with OsO_4 were not the same: mvb vesicles stained with OsO_4, whereas the inner Golgi vesicles, reactive for AcPase (28) or TPPase, did not.

While the present observations indicate that the mvb vesicles have properties in common with Golgi vesicles, they do not give any information as to what function the mvb vesicles might serve, since the mechanism of OsO_4-staining is not known. Since practically all lysosomal hydrolases have an acid pH optimum (1), one speculation is that the mvb vesicles contain enzymes or other substances responsible for acidification of the mvb. As Coffey and de Duve suggested (29), mucopolysaccharides, for example, could serve this function. Studies to explore this possibility are in progress.

Mechanism of mvb Formation

Four hypotheses have been proposed to explain the origin of the mvb. It has been suggested that they form by the penetration of vesicles through the membrane of a phagolysosome (27, 31). This phenomenon has yet to be convincingly demonstrated and receives no support from the present work. Another theory is that the mvb forms by autophagy of clusters of vesicles: smooth-membraned cisternae segregate small areas of cytoplasm that contain smooth-surfaced vesicles (19, 30, 32, and 33) and the membranes subsequently "compact" (19, 22, 34) into a single limiting membrane. Our observations of the encirclement of clusters of OsO_4-stained vesicles by smooth-membraned cisternae are consistent with this theory. Furthermore, we now have some evidence of the source of these encircled vesicles. The third theory, which has been proposed in combination with both of the above concepts and independent of them (2, 30, 35, 36), is that vesicles are added to those vesicles within the mvb by a process of budding from the mvb limiting membrane. Such vesicles would contain cytoplasmic matrix and may represent the few which did not stain with OsO_4 in this study. Lastly, Holtzman and Dominitz (36) suggest that some mvb may arise from "cup-like" bodies which participate in heterophagy. This process was not observed in the epididymis.

In summary, the findings indicate that vesicles found within mvbs are similar to outer Golgi vesicles and differ in their structure and cytochemistry from endocytic and primary lysosomal vesicles. The findings are compatible with the view that mvbs form by the encirclement of OsO_4-positive, outer Golgi vesicles by smooth-membraned cisternae which compact into a limiting membrane, thereby forming a vacuole recognized as an mvb. Other vesicles which do not stain with OsO_4 may then be added to the mvb vesicle population by budding of the mvb membrane.

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