FUNCTIONS OF COATED VESICLES DURING PROTEIN ABSORPTION IN THE RAT VAS DEFERENS

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ABSTRACT

The role of coated vesicles during the absorption of horseradish peroxidase was investigated in the epithelium of the rat vas deferens by electron microscopy and cytochemistry. Peroxidase was introduced into the vas lumen in vivo. Tissue was excised at selected intervals, fixed in formaldehyde-glutaraldehyde, sectioned without freezing, incubated in Karnovsky's medium, postfixed in OsO_4, and processed for electron microscopy. Some controls and peroxidase-perfused specimens were incubated with TPP,[1] GP, and CMP. Attention was focused on the Golgi complex, apical multivesicular bodies, and two populations of coated vesicles; large (>1000 A) ones concentrated in the apical cytoplasm and small (<750 A) ones found primarily in the Golgi region. 10 min after peroxidase injection, the tracer is found adhering to the surface plasmalemma, concentrated in bristle-coated invaginations, and within large coated vesicles. After 20–45 min, it is present in large smooth vesicles, apical multivesicular bodies, and dense bodies. Peroxidase is not seen in small coated vesicles at any interval. Counts of small coated vesicles reveal that during peroxidase absorption they first increase in number in the Golgi region and later, in the apical cytoplasm. In both control and peroxidase-perfused specimens incubated with TPP, reaction product is seen in several Golgi cisternae and in small coated vesicles in the Golgi region. With GP, reaction product is seen in one to two Golgi cisternae, multivesicular bodies, dense bodies, and small coated vesicles present in the Golgi region or near multivesicular bodies. The results demonstrate that (*a*) this epithelium functions in the absorption of protein from the duct lumen, (*b*) large coated vesicles serve as heterophagosomes to transport absorbed protein to lysosomes, and (*c*) some small coated vesicles serve as primary lysosomes to transport hydrolytic enzymes from the Golgi complex to multivesicular bodies.

INTRODUCTION

The mechanisms involved in the absorption of protein have been explored at the ultrastructural level in several tissues, notably the epithelium of the vertebrate nephron (1–9) and epididymis (10), and in insect (11, 12) and guppy (13) oocytes. It is now clear that cells which actively absorb protein do so by amplification of the general process of "heterophagy" (14), by which cells take up and degrade large molecules. This is accomplished by

[1] The following abbreviations are used in this paper: AcPase, acid phosphatase; CMP, cytidine 5′-monophosphate; ER, endoplasmic reticulum; GP, Na β-glycerophosphate; TPP, thiamine pyrophosphate; TPPase, thiamine pyrophosphatase.

incorporation of protein within membrane pockets (pinocytosis) followed by segregation and digestion within lysosomes. Whereas the main pathways for absorbed protein are clear, many of the associated events, such as the implied concomitant centrifugal circulation of membrane and enzymes, remain to be clarified.

We were particularly interested in exploring the modes of transport or connections which exist between the Golgi complex, lysosomes, and the cell surface in the intact absorptive cell. For this purpose, we applied a combined morphological and cytochemical approach, by using peroxidase as a tracer for protein absorption, and thiamine pyrophosphatase (TPPase)[1] and acid phosphatase (AcPase) as markers for Golgi membranes and lysosomes, respectively. Because our interest required an absorptive cell with a well-developed Golgi complex, we initially chose to study the epididymis (15, 16), for its Golgi complex, composed of numerous stacks of cisternae and associated vesicles, has been isolated and partially characterized biochemically (17) as well as histochemically (18, 19). It proved technically difficult, however, to avoid random diffiusion of tracer in this tissue. We therefore shifted our attention to the vas deferens which proved particularly advantageous for our purposes since tracer and fixative solutions can be introduced directly into its lumen, and since the epithelium of this structure in the rat resembles that of the epididymis in its fine structure and absorptive function (20).

This paper reports our findings on the pathway of peroxidase absorption in the vas deferens and on the role of two populations of coated vesicles, multivesicular bodies, and the Golgi complex in the absorption process.

MATERIALS AND METHODS

The tissue studied was the lining epithelium of the rat vas deferens. 3 doz mature male, Sprague-Dawley rats were used in these experiments.

Reagents and substrates were obtained from Sigma Chemical Co. (St. Louis, Mo.) as follows: GP[1] (grade I), CMP (disodium salt), TPP (cocarboxylase), 3,3′-diaminobenzidine tetrahydrochloride, (type II), and horseradish peroxidase (type II).

Experimental Procedures

EXPOSURE OF THE VAS DEFERENS: Animals were lightly anesthetized with ether and/or sodium pentabarbital, their scrota were incised, and an incision was made into the proximal (prostatic) end of the vas deferens. This permitted egress of the seminal fluid, and of the fixative and peroxidase solutions which were subsequently introduced into the duct lumen distally as described below.

PEROXIDASE EXPERIMENTS: A 6% solution of peroxidase, prepared in isotonic saline with 2–5% sucrose, was injected slowly into the duct lumen at a point approximately 1 cm from the tail of the epididymis. A continuous flow was maintained for several minutes, either manually or with a Harvard infusion pump, until ½ cc (30 mg) had been injected. The vas deferens was fixed at intervals of 7–45 min after initiation of the peroxidase injection.

Techniques for Morphological Studies

FIXATION: Fixation was initiated by injection of cold fixative into the deferential vein and the vas lumen. The fixative generally employed was a dilute version of that introduced by Karnovsky (21) and contained 1–2% paraformaldehyde and 3% glutaraldehyde in 0.067 M cacodylate buffer. The glutaraldehyde was purified by distillation prior to use as described in reference 22. After several minutes of fixation *in situ*, the middle third of the vas deferens was excised, cut into small blocks, and immersed in fresh fixative for 6 hr. In several cases fixation was carried out for 2 hr in 1% OsO_4 buffered at pH 7.4 with acetate-Veronal or *s*-collidine (23) containing 4.5% sucrose.

SUBSEQUENT PROCESSING: After fixation, the tissue blocks were washed overnight in 0.05 M acetate–Veronal buffer (pH 7.4) with 7% sucrose (hereafter referred to as Michaelis wash buffer). They were then postfixed at 4°C for 1½-2 hr in *s*-collidine-buffered OsO_4. Some blocks were treated at room temperature with 0.5% uranyl acetate in acetate–Veronal buffer prior to dehydration (24). All tissues were rapidly dehydrated in ethanol and propylene oxide and embedded in Epon 812 (25).

Sections 1 μ thick were cut from Epon blocks, affixed to glass slides, stained in 1% toluidine blue in borax (26), mounted in Krylon (Krylon, Inc., Norristown, Pa.), and examined by light microscopy.

Thin sections, cut on Porter-Blum microtomes with diamond knives, were picked up onto grids covered with a Formvar film reinforced by carbon. The mounted sections were stained with lead salts (Millonig's or Karnovksy's "A") or doubly stained with uranyl acetate followed by lead.

Techniques for Cytochemical Studies

FIXATION: Fixation was carried out in paraformaldehyde–glutaraldehyde as described for morphological studies except that the time was reduced to 4 hr

for peroxidase-injected specimens and to 3 hr in the case of tissues incubated for AcPase or TPPase.

PREPARATION OF NONFROZEN SECTIONS: Nonfrozen sections were cut at 10–30 μ on a Smith-Farquhar tissue sectioner (27), and washed overnight in Michaelis wash buffer.

PEROXIDASE METHOD: Nonfrozen sections were incubated with agitation for 20–30 min at 25°C in 10-ml aliquots of Karnovsky's medium (28, 7). The latter was prepared by adding 5 mg of diaminobenzidene and 0.01–0.02% H_2O_2 to 10 ml of 0.05 M Tris–HCl buffer (pH 7.6). Controls consisted of incubations in which substrate or H_2O_2 was omitted from the medium. The latter two procedures totally suppressed the reaction.

THIAMINE PYROPHOSPHATASE METHOD: Incubations were carried out for 1½–2 hr in the modified Wachstein-Meisel medium suggested by Novikoff and Goldfischer (18): 2 mM TPP, 5 mM $MnCl_2$, 3.6 mM $Pb(NO_3)_2$, 80 mM Tris-maleate buffer, pH 7.2. Controls consisted of incubations with TPP omitted, or with 0.01 M NaF added to the medium, or the reaction carried out at pH 5. The latter two procedures did not significantly affect the localization or intensity of the reaction.

ACID PHOSPHATASE METHOD: Some sections were incubated at pH 5 for 1½–2 hr in the Barka-Anderson modification (29) of the Gomori medium with GP as substrate. Other sections were incubated with CMP as substrate and manganese as activating ion as suggested by Novikoff (30): 3 mM CMP, 3.6 mM $Pb(NO_3)_2$, 20 mM acetate buffer, pH 5.0. Controls consisted of incubations in which GP or CMP were omitted, or 0.01 M NaF was added to the medium, or the incubation was carried out at pH 7.2. All these procedures totally suppressed the reaction.

PREPARATIVE PROCEDURES FOR LIGHT AND ELECTRON MICROSCOPY: After incubation, sections were rinsed twice for 5 min in Michaelis wash buffer. For light microscopy, 30-μ sections incubated for peroxidase were mounted in glycerogel and were examined directly; similar sections incubated for TPPase and AcPase were treated with dilute $(NH_4)_2S$ before mounting. For electron microscopy, 30-μ incubated sections were postfixed at 4°C for 45 min in *s*-collidine-buffered OsO_4. Some sections were treated for 45 min with 0.5% uranyl acetate in acetate-Veronal buffer prior to dehydration as described for morphological studies. This procedure not only acts as a membrane stain, but also serves to remove some of the diffuse, presumably nonspecific reaction product (22).

Sections 0.5–1 μ were also routinely cut, affixed to glass slides, and examined by direct light or phase-contrast microscopy in order to evaluate the amount and distribution of the reaction product. The distribution of peroxidase reaction product was determined directly in unstained sections or those lightly stained with toluidine blue as described for morphological studies. The distribution of reaction product with TPP, GP, or CMP was determined in sections treated for 30 min with 2% $(NH_4)_2S$ before staining to convert colorless lead phosphate to brown lead sulfide.

Electron Microscopy

Micrographs were taken at magnifications of 4,600–30,000 on a Siemens Elmiskop I, operating at 80 kv, with a double condenser and a 50 μ molybdenum aperture in the objective.

Vesicle Counts

The numbers of small coated vesicles were counted on 8 × 11 inch prints prepared at a final magnification of 46,000. Small coated vesicles in continuity with smooth membranes and those free in the cytoplasm were included in the counts. The Student's *t*-test and tables of *P*-values were employed for evaluation of statistical significance.

OBSERVATIONS

Cytology of the Epithelium of the Vas Deferens

The fine structural organization of the principal cells of the rat vas deferens has been described by Niemi (20). Accordingly, only those structural features pertinent to this study, namely the organization of the surface membrane, lysosomes, Golgi complex, and populations of coated vesicles, will be described in detail.

GENERAL DESCRIPTION: The lining of the vas deferens consists of a layer of tall, columnar epithelium composed of principal cells along with a few dark, mitochondria-rich "pencil cells" (20) (Fig. 1). The latter are definitely minority elements and will be described in detail in a separate paper. The principal cells, with which we are exclusively concerned in this study, can be divided into three main zones (Fig. 2): a basal zone in which the nucleus and concentrations of smooth and rough surfaced ER are located; a middle region containing a large, prominent Golgi complex; and an apical zone containing numerous vesicles, vacuoles, multivesicular bodies, and microvilli ("stereocilia") (15, 16) which project into the vas lumen.

APICAL CELL SURFACE: As in the case of other lumen-lining epithelia (31), the apical plasmalemma covering the microvilli is thicker (100 A) than that along the lateral and basal cell surfaces (75 A). It lacks the rich coating of filamentous knap or "fuzz" seen along the apical surfaces of gastric and intestinal epithelia (32). Numerous

invaginations are found at the base of the microvilli, some of which have prominent bristle coats (11) on their cytoplasmic surface (Fig. 3). Just beneath this surface membrane are found some large (> 1000 A), spherical, bristle-coated vesicles (Fig. 3) and numerous, spherical and elongate, smooth-surfaced vesicles. The membrane of the large apical vesicles has the same thickness as that of the apical cell surface.

LYSOSOMES: The most commonly encountered lysosomes are large, spherical, multivesicular bodies which occur either singly or in clusters of two to three in the apical cytoplasm. Typically these structures contain relatively few vesicles in a matrix of low density (Figs. 4 and 5) and a clump of finely particulate material (Fig. 5). Both the limiting membrane of the bodies and that of its contained vesicles are of the thicker (100 A) variety like the apical cell membrane. Frequently, plaques are seen along the cytoplasmic surface of the limiting membrane (Fig. 4). Sometimes the plane of section does not include any of the internal vesicles, but these bodies are nonetheless distinguishable by their size, their thick membrane, and the clumps of particulate material present in their matrix. Occasional images are encountered which suggest that the contained vesicles are formed by invagination of the body's limiting membrane (Fig. 20). Smaller multivesicular bodies, less regular in contour, with more vesicles and a denser matrix (Fig. 6) are sometimes seen in the Golgi region. A few dense bodies are commonly present, primarily in the Golgi and basal regions.

GOLGI COMPLEX: The Golgi complex consists of abundant stacks of cisternae and associated vesicles forming a supranuclear collar (Figs. 2, 7, and 28). Each stack is composed of six to eleven cisternae with an inner, concave surface and an outer, convex surface. Other smooth-surfaced cisternae usually occur in the cytoplasmic core circumscribed by the Golgi stacks. These have a random orientation and tend to occur singly rather than in stacks; thereby they resemble similar structures described as "GERL" by Novikoff and his co-workers in

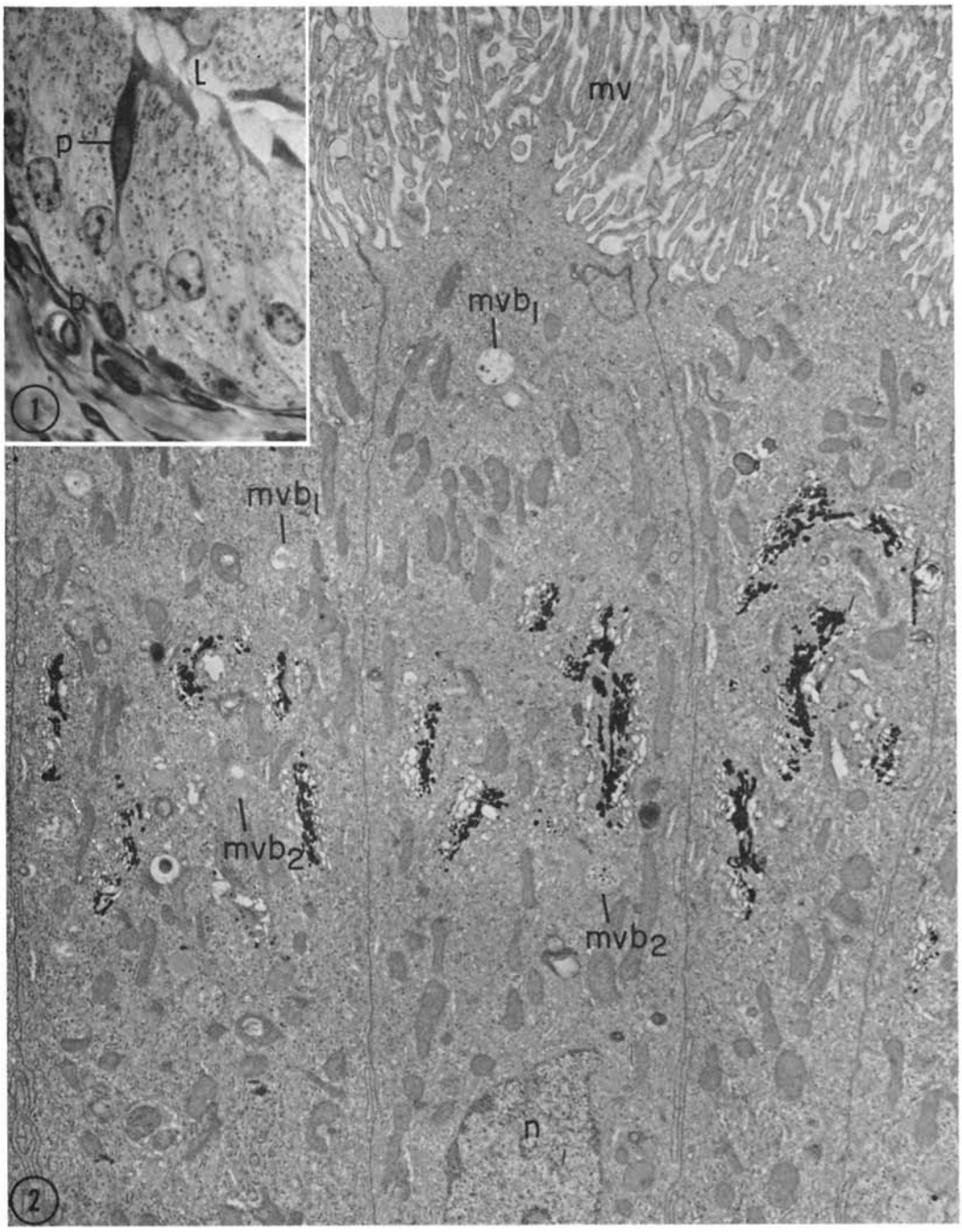
Key to Symbols

b, basement membrane	*lv*, large coated vesicle
cm, cell membrane	*mv*, microvilli
d, dense body	*mvb*, multivesicular body
Gc, Golgi complex	*n*, nucleus
ip, intermicrovillar pit	*p*, pencil cell
l, lumen	*sv*, small coated vesicle

FIG. 1 is from rat vas deferens fixed in OsO_4 buffered with *s*-collidine. Figs. 2 and 11–40 are from 10–30-μ nonfrozen sections fixed in paraformaldehyde–glutaraldehyde and incubated in appropriate media as indicated. Figs. 3–10 were prepared from 1 mm^3 tissue blocks fixed in paraformaldehyde–glutaraldehyde. In all cases except Fig. 1, the tissue was treated with uranyl acetate prior to dehydration, and sections were doubly stained with uranyl and lead.

FIGURE 1 Photomicrograph of the rat vas deferens, showing its tall columnar lining epithelium composed primarily of principal cells with long microvilli which extend into the patent lumen (*l*). Occasional deeply osmiophilic pencil cells (*p*) are also present. A moderately thick basement membrane (*b*) separates the epithelium from its underlying vasculature, connective tissue, and smooth muscle tunic. × 700.

FIGURE 2 Several epithelial cells from the rat vas deferens are shown in this low power electron micrograph. Numerous microvilli (*mv*) are seen along the apical cell surface and several multivesicular bodies (mvb_1) are present in the apical cytoplasm. The Golgi apcomplex, which consists of five to six groups of stacked cisternae, is located midway between the cell surface and the nucleus (*n*). The cisternae are marked by dense deposits of lead-phosphate reaction product in this specimen which was incubated with TPP. Several multivesicular bodies (mvb_2) occur also in the Golgi region and mitochondria are distributed throughout the cytoplasm. × 8,000.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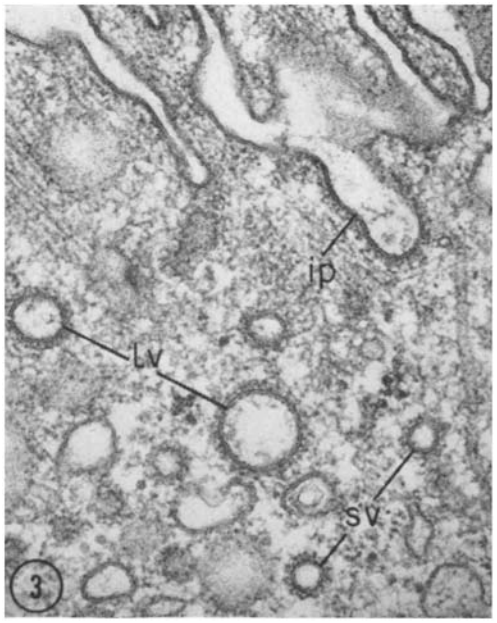
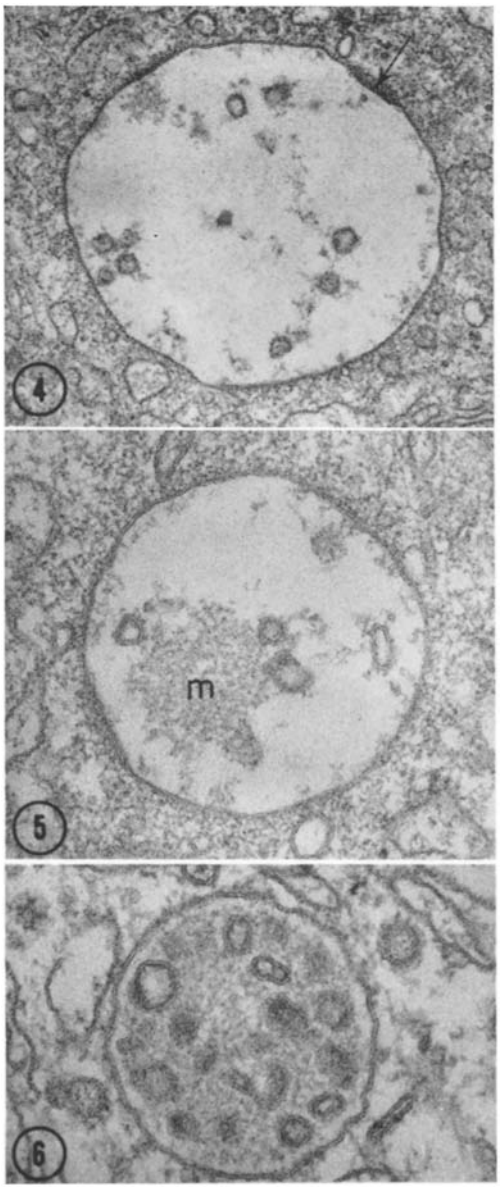


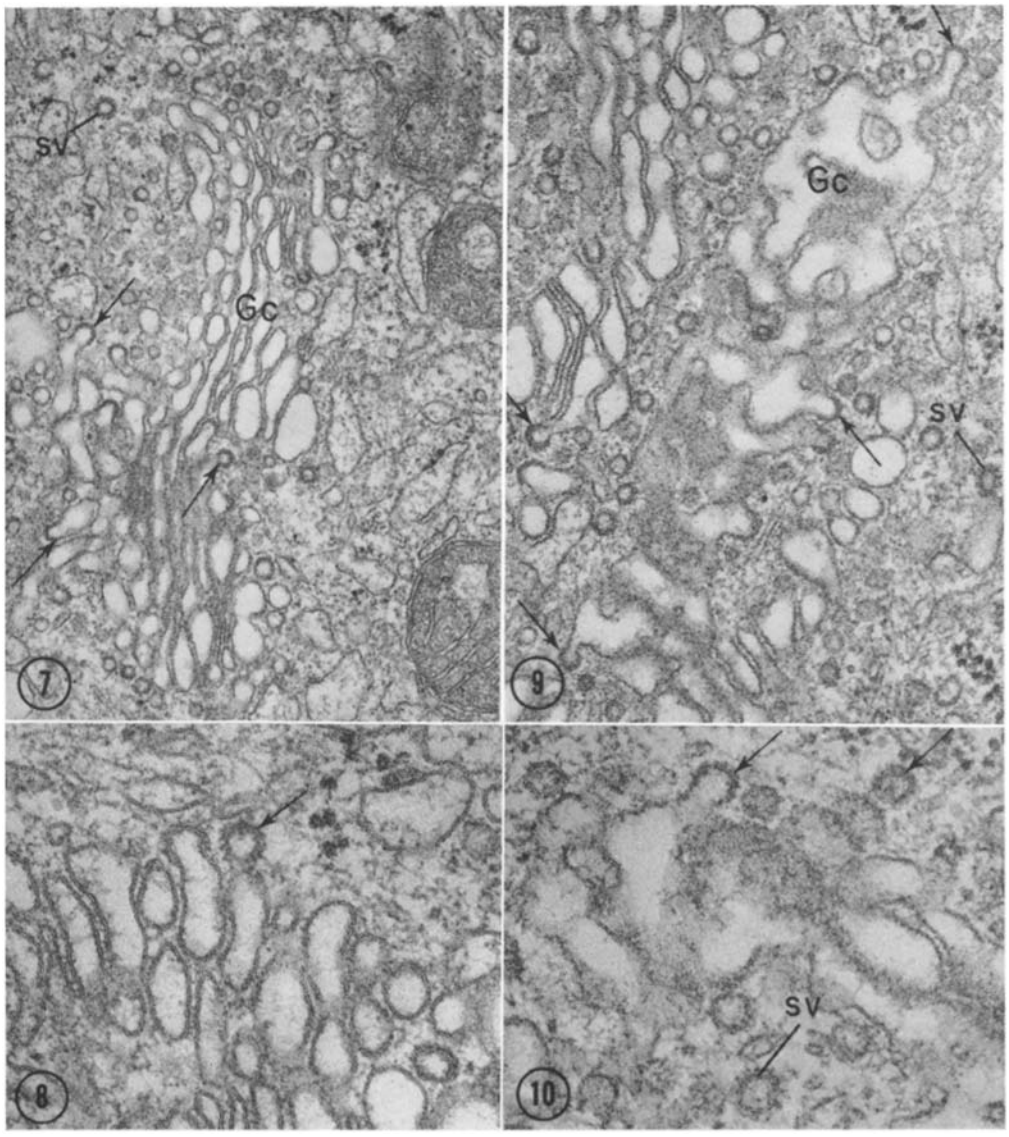
FIGURE 3 Portion of the apical epithelial cell surface showing intermicrovillar pits (*ip*) and two sizes of coated vesicles: large ones (*lv*), greater than 1000 A in diameter, and smaller ones (*sv*) measuring 600–700 A. The intermicrovillar pit shows a bristle coat similar to that of the vesicles, on its cytoplasmic surface. × 72,000.

neurons of rat spinal cord (33) and parenchymal cells of the liver (34). The majority of the vesicles associated with the cisternae are smooth-surfaced and measure 600 A in diameter, but 10–20% of those present are of the coated variety and measure 750 A (Figs 7–10). No secretory product is discernible in the vacuoles or cisternae of the Golgi complex.

COATED VESICLES: As can be seen from the foregoing, two types of coated vesicles are evident in these cells : (*a*) large ones, 1000 A or more in diameter, found near the cell surface, and (*b*) smaller ones, 750 A, which are concentrated near the Golgi complex. It is of interest that the content of the smaller coated vesicles is generally more granular and denser than that of the larger ones. Those of both types have a coat with equidistant, radially arranged bristles, 150–200 A in length, extending from the outer leaflet of their limiting membrane; 18–25 bristles can be counted around the larger coated vesicles and 8–13 around the smaller ones. The bristles represent contiguous, parallel sides of irregular hexagons which cover



FIGURES 4–6 Multivesicular bodies from controls. Those found in the apical cytoplasm (Figs. 4–5) tend to be larger, contain fewer vesicles, and have a lighter background matrix than those present in the Golgi region (Fig. 6). A moderately dense, finely granular mass (*m*) is often found in the matrix of those in the apical cytoplasm, and occasionally plaques (arrow, Fig. 4) are present along the outer surface of their limiting membrane. Fig. 4, × 50,000; Fig. 5, × 78,000; Fig. 6, × 90,000.



FIGURES 7 and 8 Low and high magnification micrographs, respectively, of the Golgi complex (*Gc*) from control preparations sectioned in a plane perpendicular to the long axis of the Golgi cisternae. Numerous continuities between small coated vesicles and Golgi cisternae are evident (arrows) and several coated vesicles (*sv*) are seen nearby. Fig. 7, × 40,000; Fig. 8, × 93,000.

FIGURES 9 and 10 Similar fields of the Golgi complex (*Gc*) sectioned parallel to the long axis of the cisternae with several examples (arrows) of continuities between small coated vesicles (*sv*) and Golgi cisternae. Fig. 9, × 53,000; Fig. 10, × 90,000.

the surface of the unit membrane (35). Each wall of the polygon is about 30 A in thickness, but it is not ordinarily preserved sufficiently to be seen as an individual unit. Two sides together, however, appear as a "bristle." As already indicated, the large coated vesicles are seen exclusively near the apical cell surface. The smaller ones are most numerous in the Golgi zone and occasionally can

be seen in continuity with Golgi cisternae (Figs. 7–10) or with the smooth-surfaced cisternae in the Golgi cytoplasmic core. However, individual, small coated vesicles can be found distributed throughout the cytoplasm, particularly adjacent to lytic bodies and near the apical and lateral portions of the cell membrane (Fig. 3).

Peroxidase Absorption

10 MIN: At 10 min after peroxidase injection the dense tracer material is present (*a*) in the lumen between the microvilli (*b*) concentrated in intermicrovillar pits, and (*c*) in some of the large coated vesicles near the apical cell surface, (Figs. 11–14). The heaviest concentrations of peroxidase are seen in the cul de sacs at the deepest end of the invaginations (Fig. 12). Small areas devoid of peroxidase are occasionally seen in the neck region of the invagination above such cul de sacs, and rarely, small coated vesicles are found in continuity with the cell membrane in these clear regions (Fig. 25).

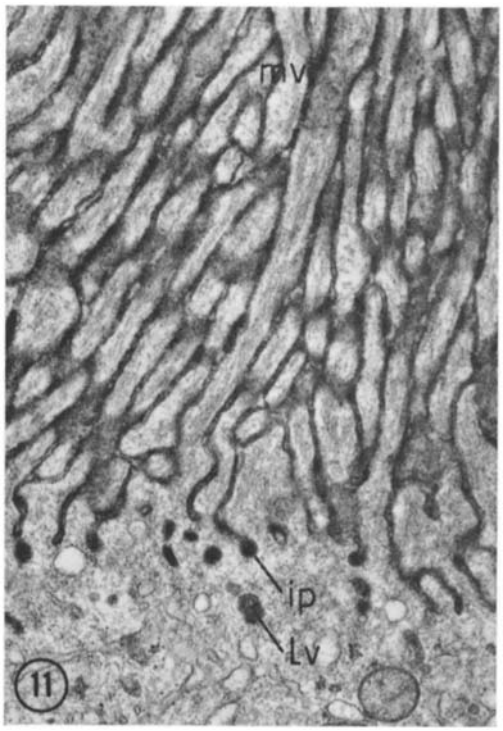


FIGURE 11 Portion of the apical cell surface from a specimen fixed 10 min after peroxidase injection. Dense deposits of peroxidase reaction product are seen covering the microvilli (*mv*), in the intermicrovillar pits (*ip*), and in large apical vesicles (*lv*). × 20,000.

At this time increased numbers of small coated vesicles are seen in the Golgi region (Fig. 22). Results of vesicle counts (Table I) reveal that there are roughly twice the number seen in comparable areas of control preparations. None of these small coated vesicles contains peroxidase.

20 MIN: In addition to the sites described at earlier intervals, peroxidase is seen in smooth apical vesicles and in some of the apical multivesicular bodies (Figs. 15 and 16), and occasional images reveal continuity between the limiting membranes of these two structures, their peroxidase content confluent (Figs. 17 and 18). Within multivesicular bodies, peroxidase is found throughout the matrix but is not present within the contained vesicles (Figs. 16, 18, and 21).

At this interval, streams of small coated vesicles which do not contain peroxidase are seen between the Golgi complex and the apical surface.

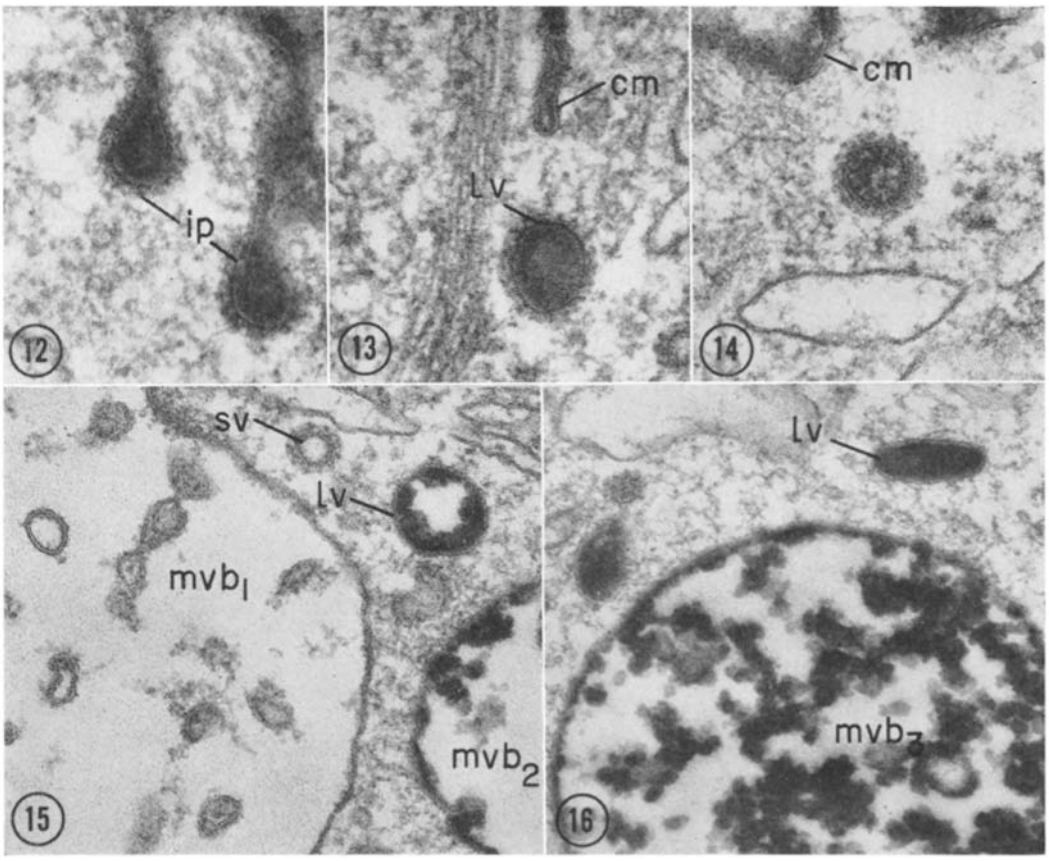
40 MIN: By this time, the majority of the multivesicular bodies are engorged with peroxidase (Fig. 19), and dense bodies are more numerous than in the controls.

The frequency of small coated vesicles in the Golgi region is comparable to that of the controls, but increased numbers are found in the apical cytoplasm (Table I). Clusters of small coated vesicles are commonly encountered surrounding the peroxidase-containing multivesicular bodies (Fig. 23) and beneath the apical cell membrane (Figs. 24–26) but, as at earlier time points, these vesicles do not contain peroxidase. It is of interest that, coincident with the appearance of small coated vesicles near the cell surface, small patches devoid of peroxidase can be seen along the apical cell membrane more frequently than at 10 min; this suggests that some of the small, peroxidase-free vesicles have fused with the surface membrane (Figs. 25 and 26).

Peroxidase is not seen in the cisternae of the ER or Golgi complex at this or any time interval. Furthermore, with the exception of mechanically traumatized areas, it is not seen between cells (Fig. 27, inset).

Results of Vesicle Counts

Counts were made of the numbers of small coated vesicles present in 160 randomly selected principal cells of the vas deferens. The data were compiled from four different experiments involving eight rats, four peroxidase-perfused, and four controls. 20 cells were counted from each animal



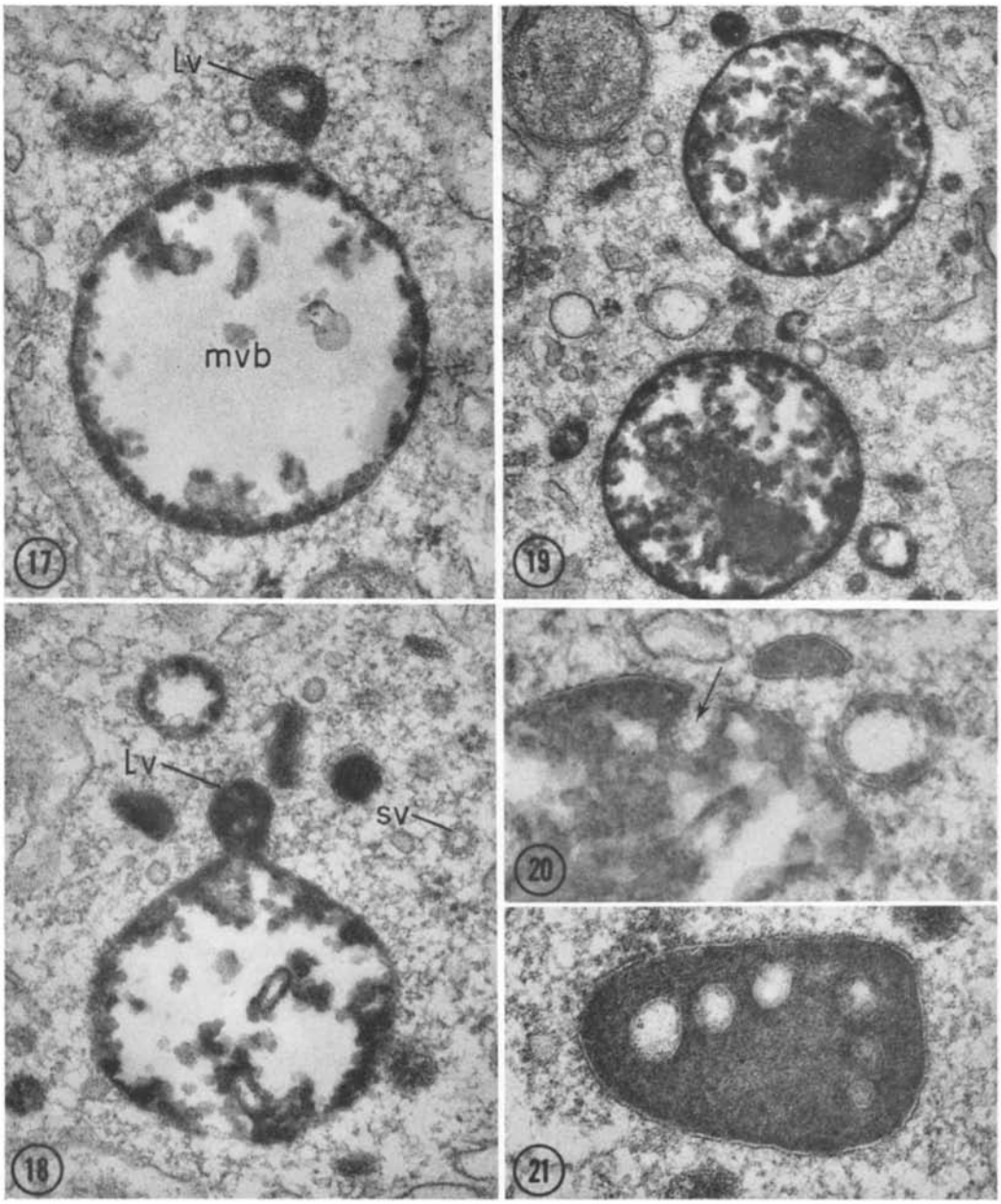
FIGURES 12–16 Higher magnification fields from peroxidase-perfused specimens. At 10 min the tracer is seen in bristle-coated intermicrovillar pits (*ip*) (Fig. 12) and in large coated vesicles (*lv*) located near the apical cell membrane (*cm*) (Figs. 13–14). After 20 min peroxidase is found in large, spherical (Fig. 15) or elongate (Fig. 16) smooth vesicles (*lv*) which have apparently lost their bristle coat and lie near multivesicular bodies (*mvb*). Some of the latter (mvb_2 and mvb_3) also contain peroxidase. In Fig. 15, a multivesicular body which contains the tracer (mvb_2) is present alongside another (mvb_1) which does not. Small coated vesicles (*sv*) are also frequently seen in the vicinity of multivesicular bodies (Fig. 15), but they do not contain the tracer. Figs. 12–14, × 100,000; Fig. 15, × 88,000; Fig. 16 × 78,000.

and one or more fields through the Golgi or apical regions were counted per cell. A total of 1,134 vesicles were counted.

The results (Table I) reveal that small coated vesicles in the Golgi region increase from an average of 7 in controls to 18 per field at 10 min after peroxidase injection. At 40 min their number significantly decreases ($P < 0.005$) from 18 to 8. Congruently, by 40 min, the number of small coated vesicles has increased in the apical cytoplasm from 8 to 16. It is of interest that the increase in small coated vesicles found in the apical cytoplasm is comparable in magnitude to the decrease in small coated vesicles found in the Golgi region at the same time period. P values are < 0.005 between each time interval.

Thiamine Pyrophosphatase Localization

CONTROLS: In specimens incubated with TPP, the first four to five cisternae along the inner, concave surface of the Golgi complex are strongly positive (Figs. 1, 28, and 29). A decreasing intensity gradient is sometimes evident, proceeding from the first through the fourth and fifth cisternae. Deposits are rarely present in the single, smooth cisterna (GERL) of the Golgi core.



FIGURES 17–21 Small fields from peroxidase-perfused preparations. Figs. 17–18, taken at 20 min, show what appear to represent progressive stages in the mergence of large vesicles (*lv*) with multivesicular bodies (*mvb*). A small coated vesicle (*sv*) which does not contain peroxidase is seen near the multivesicular body in Fig. 18. Fig. 19, taken at 40 min, shows several multivesicular bodies engorged with peroxidase; the reaction product adheres to the inner leaflet of the limiting membrane, covers the fibrillar material in the matrix, and loosely fills the formerly clear portion of the matrix. Several large vesicles marked with peroxidase, are also present. Fig. 20 shows an invagination of the limiting membrane of a multivesicular body (arrow). Such images suggest that the internal vesicles may be formed by pinching off from the body's membrane. Fig. 21 depicts a dense body which was presumably formed by filling of a multivesicular body with peroxidase, followed by condensation of its content. The internal vesicles, which do not contain peroxidase, stand out sharply against the dark background provided by the dense matrix. Fig. 17, × 65,000; Fig. 18, × 70,000; Fig. 19, × 38,000; Figs. 20 and 21, × 90,000.

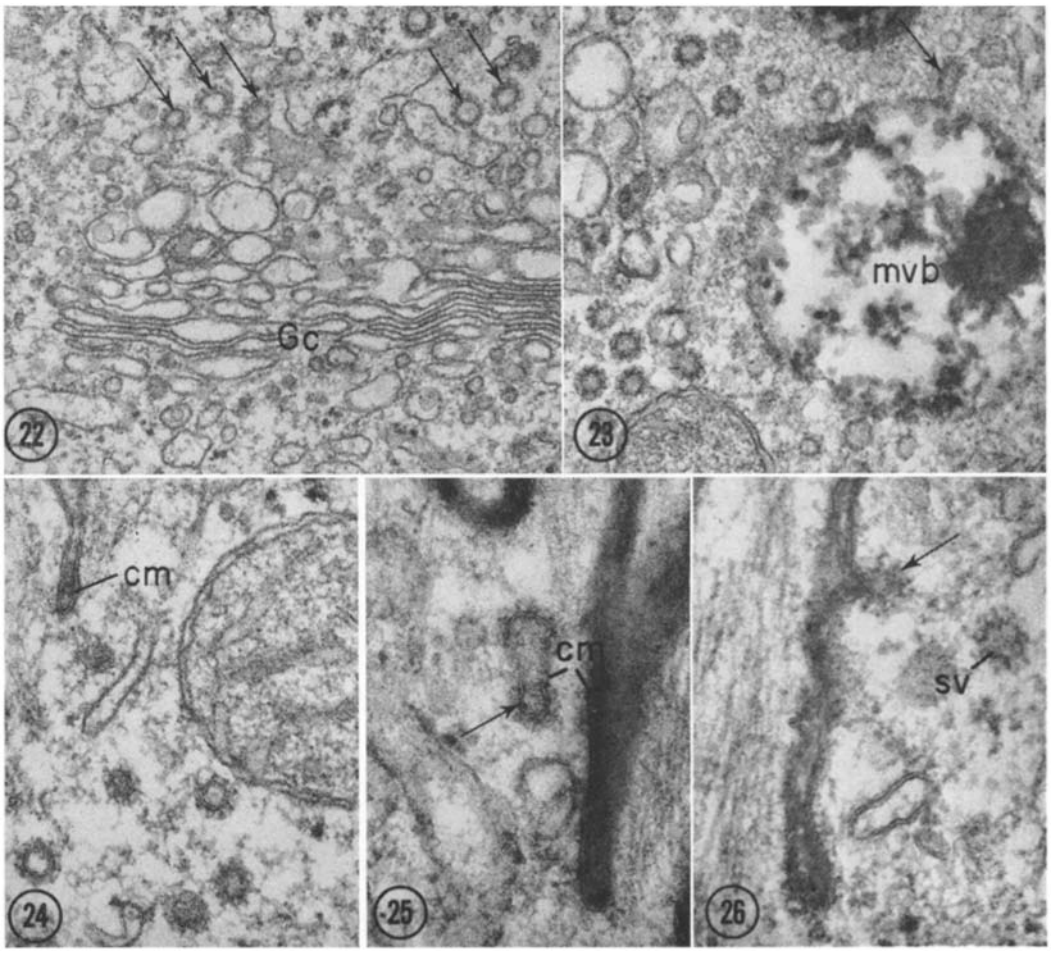


FIGURE 22 Golgi cisternae from a specimen fixed 10 min after peroxidase injection. The number of small coated vesicles (arrows) found in the Golgi region is greater than in controls. × 43,000.

FIGURE 23 Peroxidase-perfused specimen (40 min). Large numbers of small coated vesicles are present around a peroxidase-filled multivesicular body (*mvb*). An image suggesting fusion between a small coated vesicle and a multivesicular body is seen (arrow). × 52,000.

FIGURES 24–26 At later time points (40 min), large numbers of small coated vesicles are present beneath the apical cell membrane (*cm*) (Fig. 24). In addition, clear patches devoid of peroxidase are seen in invaginations of the cell membrane (Fig. 25), and occasional images are seen suggestive of fusion between the two (Figs. 25, 26; arrows). As at earlier time points, small coated vesicles (*sv*) do not contain peroxidase. Fig. 24, × 68,000; Fig. 25, × 75,000 and Fig. 26, × 100,000.

No reaction product is seen within the smooth, Golgi-associated vesicles located in continuity with, or in proximity to the Golgi cisternae, but deposits are found in small coated vesicles in the Golgi region (Figs. 28 and 29). Only rarely are deposits found in small coated vesicles remote from the Golgi region.

Large coated vesicles do not contain reaction product, and none is seen within the rough ER.

PEROXIDASE PERFUSED: With TPP, activity appears slightly increased in specimens perfused with peroxidase for 10 min, and definitely increased by 20 min; there is a more uniform reaction in the Golgi complex from cell to cell as compared to controls, and reaction product is sometimes seen in

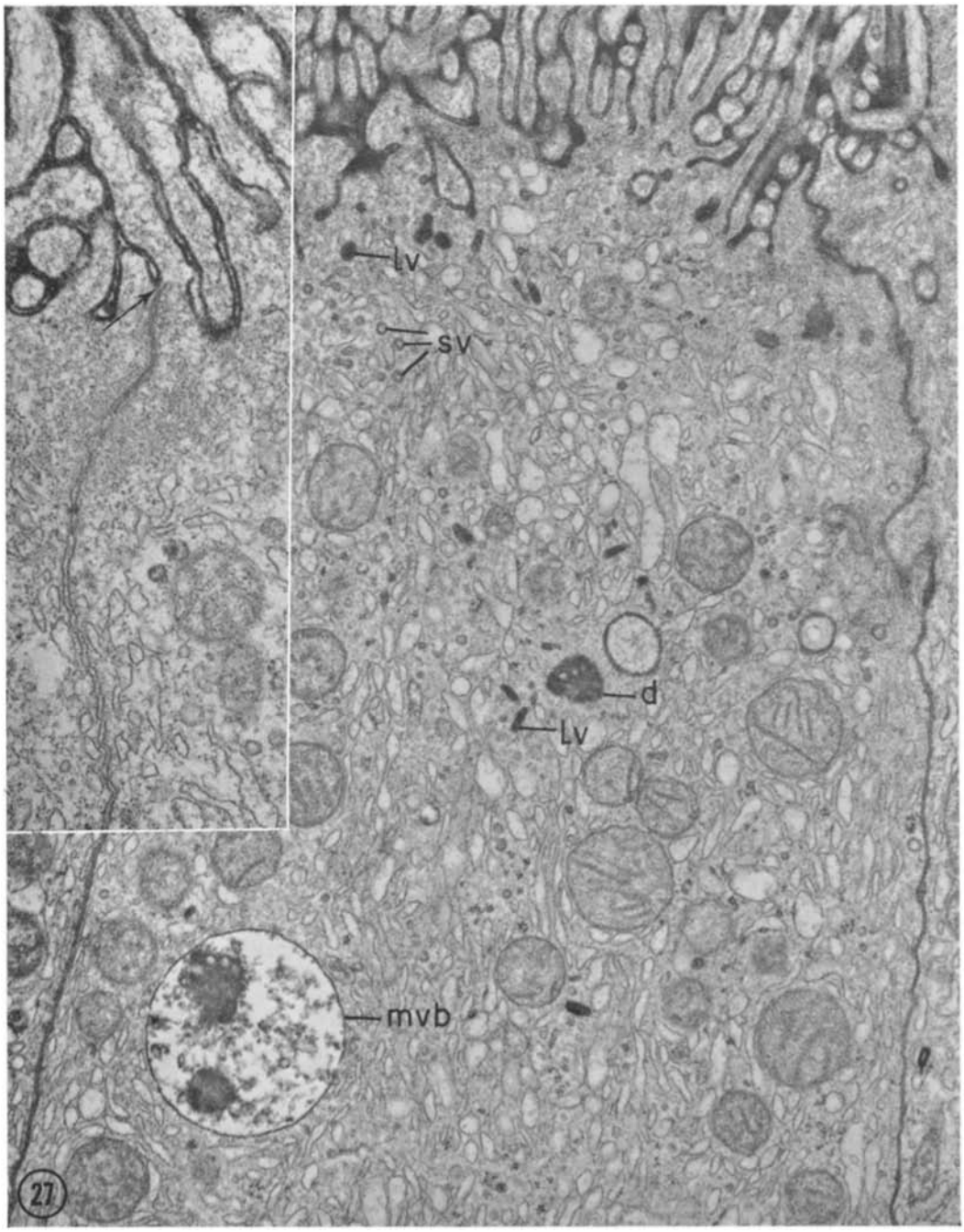


FIGURE 27 This low power micrograph summarizes the findings at 40 min after peroxidase injection. The tracer is seen in large bristle-coated invaginations of the apical cell surface which apparently migrate basally, lose their coat, and become smooth vesicles (*lv*) which eventually fuse with multivesicular bodies (*mvb*). The latter condense to form dense bodies (*d*). There is also an increase in the number of small coated vesicles (*sv*) found in the apical region. Peroxidase is not seen in small coated vesicles, the ER, or the Golgi complex. The tracer does not appear to penetrate between cells, for it is not detectable in the intercellular spaces below the level of the occluding zonules (arrow, inset). Fig. 27, × 17,000; inset × 37,000.

five or more Golgi cisternae. As in the controls, some of the small coated vesicles contain reaction product, and those that do are confined virtually exclusively to the Golgi region.

Acid Phosphatase Localization

CONTROLS: With GP as substrate, reaction product is present within the first one or two cisternae along the concave surface of the Golgi complex (Figs. 30 and 31), in some Golgi vesicles, multivesicular bodies, and dense bodies (Figs. 31–35).[2] It is also occasionally seen in the smooth

TABLE I

Average Number of Small Coated Vesicles per Field from Principal Cells of the Vas Deferens‡*

Controls		Peroxidase-perfused	
		10 min	*40 min*
Golgi Region	7(±1)§	18(±1)	8(±1)
Apex		8(±1)	16(±1)

* 8 × 11 inches print at × 46,000.
‡ For each group, 40–60 fields were counted.
§ Standard error.

cisterna of the Golgi core (GERL). In contrast to the findings with TPP, with GP the reaction product appears associated with the content rather than the membranes of these compartments. The amount of reaction product in multivesicular bodies is quite variable, but in all instances it is confined to the matrix of these bodies and is not seen within their vesicles (Figs. 34 and 35). Some small coated vesicles are seen to contain reaction product. These occur in continuity with Golgi cisternae (Fig. 31), free in the Golgi region, and in the peripheral regions of the cytoplasm adjacent to multivesicular bodies (Figs. 32–34). Unfortunately, in specimens incubated at acid pH, the bristles are frequently blurred. Hence it is difficult to determine whether all of the AcPase-positive, small vesicles are coated (Figs. 33 and 34). Activity is not found in the rough-surfaced ER in the principal cells. In addition to these sites already mentioned, heavy deposits of lead phosphate are seen along the surface of the microvilli, (Fig. 36), within some of the intermicrovillar invaginations, and very rarely in the most superficial large coated vesicles. Reaction product is *not* seen within large vesicles deeper in the cytoplasm. Clumps of reaction product are also seen adhering to sperm in the duct lumen. This extracellular reaction des not appear to be due to nonspecific alkaline phosphatase, for these sites are negative when the reaction is carried out at alkaline pH. It could be due to AcPase from the ventral prostate which is known to be rich in this enzyme.

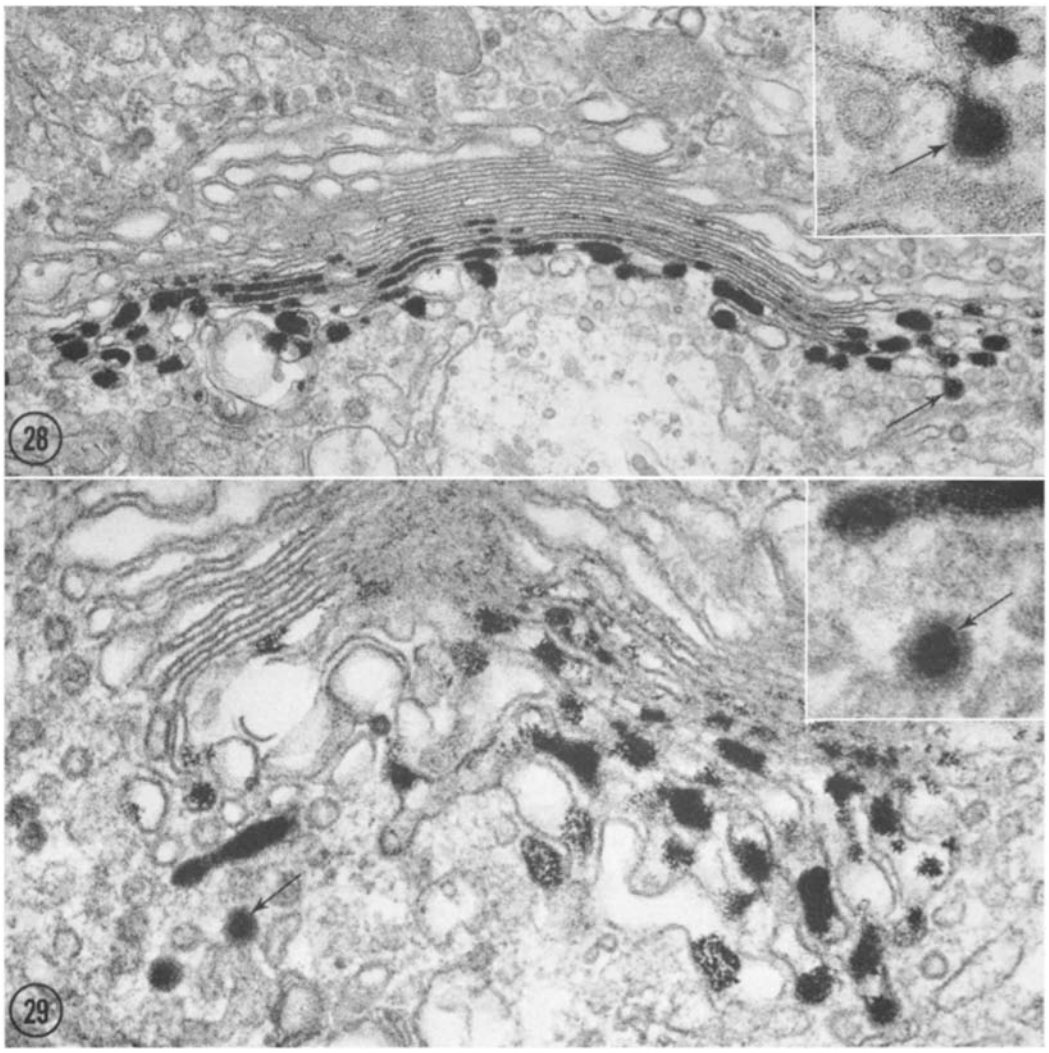
With CMP as substrate, the reaction in Golgi elements, small coated vesicles, multivesicular bodies, and dense bodies is generally the same as with GP except that the reaction in these last two sites is more uniformly distributed and consistently present. A notable difference, however, between the findings with CMP and those with GP is that with the former, no reaction product is seen along the microvilli, along the intermicrovillar invaginations, or around sperm present in the duct lumen.

PEROXIDASE PERFUSED: With GP, activity appears increased in specimens perfused with peroxidase for 20 min. Reaction product is seen in more cisternae (3–4) and in more Golgi-associated vesicles as compared to the controls (Figs. 37–39). As in the controls, however, it is difficult to distinguish small coated vesicles in preparations incubated at acid pH's. Counts of small vesicles with reaction product were therefore not made. The distribution of reaction product was otherwise similar to that described in controls.

Curiously, contrary to our findings with GP, in specimens incubated with CMP, a decrease in reaction of the Golgi cisternae is appreciable even after 10 min and is pronounced after 20 min of peroxidase perfusion (Fig. 40). Congruently, reaction product appears more extensive in multivesicular bodies and dense bodies as compared with controls. There is no appreciable difference in the number of labeled, small vesicles.

SUMMARY OF FINDINGS: Horseradish peroxidase is taken up in bristle-coated invaginations of the apical cell membrane. These invaginations pinch off and become large coated vesicles in the superficial cytoplasm. The large coated vesicles lose their coat, become smooth, and subsequently fuse with, and discharge their peroxidase content into multivesicular bodies. The peroxidase content of the latter increases as a function of time. Concomitantly, there is a doubling in the number of small coated vesicles and a shift in their distribution from

[2] Figs. 31–35 are from specimens incubated with CMP, but the findings are the same as with GP (see below).

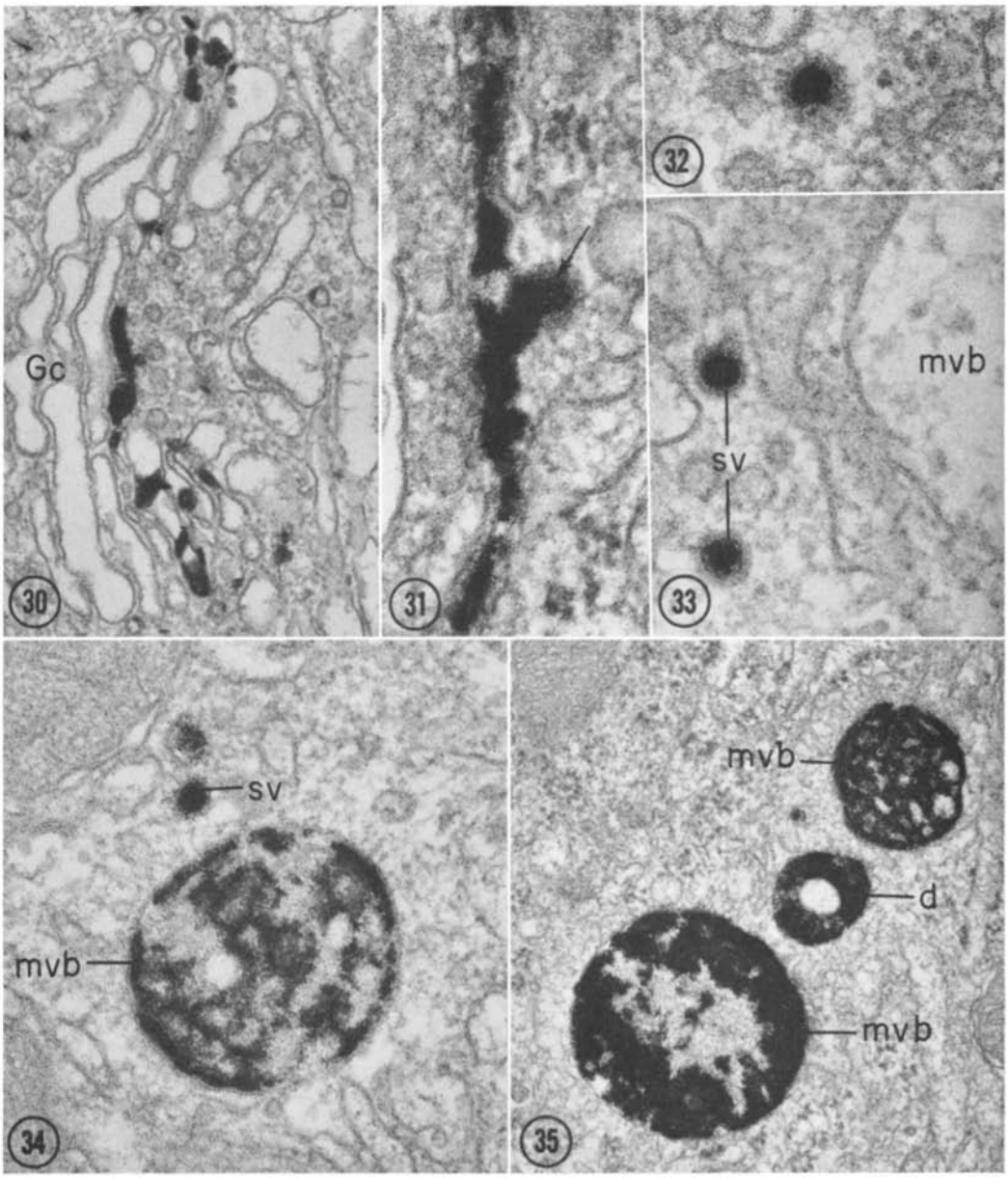


FIGURES 28–29 Golgi cisternae from a control preparation incubated with TPP. Reaction product is present in four to five cisternae along the concave surface of the Golgi apparatus, and in some small coated vesicles which are in continuity with (Fig. 28, arrow), or in close apposition to (Fig. 29, arrow) Golgi cisternae. For each figure the vesicles indicated with arrows are enlarged in the inset. Fig. 28, × 44,000; inset × 130,000; Fig. 29, × 99,000; inset × 190,000.

the Golgi region to the apical cytoplasm near multivesicular bodies and the apical cell membrane with which they apparently fuse. In both control and peroxidase-perfused specimens some of the small coated vesicles found in the Golgi region are reactive with TPP, GP, and CMP. No TPP-positive vesicles are seen elsewhere in the cell. With CMP or GP, some of those present near multivesicular bodies as well as the Golgi-associated coated vesicles are reactive. After peroxidase perfusion, activity with TPP and GP increases in the Golgi apparatus whereas that with CMP decreases; activity in multivesicular bodies and dense bodies is similar with GP and increased with CMP as compared to controls. As in the controls, some small coated vesicles in peroxidase-perfused specimens are reactive with TPP, GP, and CMP.

DISCUSSION

The results of this study have verified previous assertions that the vas deferens is absorptive in function. Furthermore, they have corroborated



FIGURES 30–31 Stacks of Golgi cisternae from a control preparation incubated with GP (Fig. 30) or CMP (Fig. 31). Lead-phosphate deposits are seen in the innermost cisterna of the stack. In Fig. 31, a small coated vesicle, filled with reaction product, is shown in continuity with the reactive cisterna (arrow). Fig. 30, × 50,000; Fig. 31, × 120,000.

FIGURES 32–35 Control preparations incubated with CMP. Reactive, small coated vesicles are present in the Golgi region (Fig. 32) and adjacent to a multivesicular body (*mvb*) (Fig. 33). Figs. 34 and 35 depict multivesicular bodies (*mvb*) and a dense body (*d*) which contain heavy deposits of reaction product. In Fig. 34 a small coated vesicle (*sv*), filled with reaction product, is seen alongside the multivesicular body. Fig. 32, × 100,000; Fig. 33, × 110,000; Fig. 34, × 93,000; Fig. 35, × 50,000.

recent work on the uptake and intracellular pathway for protein absorption. Of greater interest, is the new cytochemical and statistical evidence obtained on the direction of movement and transport functions of coated vesicles during protein absorption. Specifically, several different types of coated vesicles have been distinguished in the epithelium of the rat vas deferens: one type, the larger in diameter, is formed at the cell surface by pinocytic invagination of the apical cell membrane, moves toward and fuses with multivesicular bodies, and serves to transport absorbed protein from the duct lumen to lytic bodies. The other type of coated vesicle is smaller, originates from Golgi cisternae, and moves to a peripheral location in the cell. Some of these apparently transport AcPase, and possibly other acid hydrolases, from their site of packaging (Golgi complex) to their site of action (multivesicular body). Others apparently fuse with the surface membrane, but the nature of their content remains unknown. Thus the findings have demonstrated the existence in this epithelium of several functionally distinct types of coated vesicles.

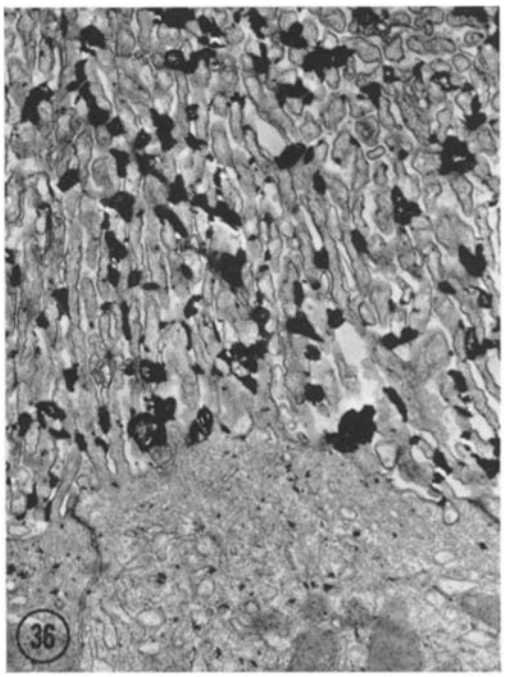


FIGURE 36 Control preparation incubated with GP. Dense clumps of reaction product are seen along the microvilli. This surface reaction is inhibited by adding NaF to, or omitting GP from, the incubation medium. It is not seen when CMP is used as the substrate or when the reaction is carried out at pH 9. × 15,000.

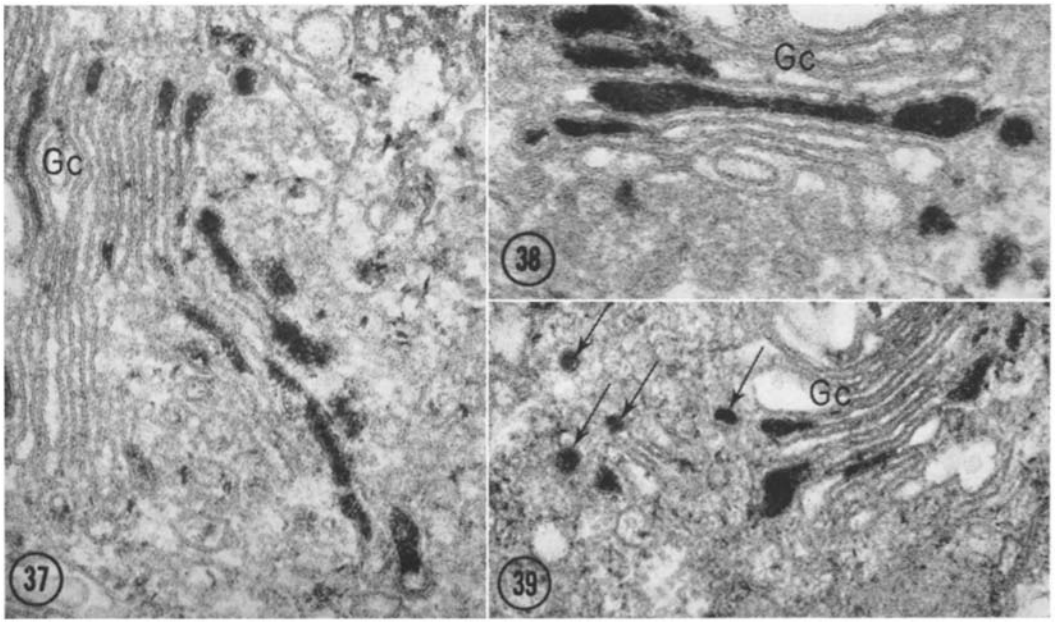
Functions of the Vas Deferens

The fine structural organization of the vas deferens has been studied previously in the rat by Niemi (20) who, noting the basic similarity of this epithelium to that of the epididymis, postulated that the vas deferens may function in pinocytosis, as had been demonstrated by Burgos (15) and Nicander (16) for the rat epididymis and Sedar (10) for the hamster epididymis. Our findings confirm the similarities in structure and histochemical activities of these two epithelia, and, in addition, have demonstrated the ability of the vas epithelium to absorb protein. It follows that, in the rat, this duct is not simply a passive conduit for transport or sperm, since it can modify the content of the semen through selective absorption.

Absorption and Heterolysis

Peroxidase seems to follow the same general pathway followed by absorbed protein in other tissues (1–13): it is taken up in coated invaginations of the absorptive cell surface, transported *via* smooth vesicles to a digestive vacuole, and subsequently degraded. This over-all process has been named "heterolysis" by de Duve and Wattiaux (14). According to their systematization, the smooth vesicle, which contains absorbed protein but no hydrolytic enzymes, is called a heterophagosome, and the digestive vacuole, with both enzyme and ingested protein, is called a heterolysosome. In the vas epithelium the large coated vesicles therefore correspond to heterophagosomes and the multivesicular bodies to heterolysosomes.

In previous work on the absorptive process, the mechanism of acquisition of lytic enzymes by digestive vacuoles has been difficult to follow. The work of Straus (4) at the light microscope level has clearly demonstrated that the phagosomes, in which absorbed protein is sequestered, acquire lytic enzymes within minutes after protein uptake, but it has been difficult to discern whether acquisition occurs by direct delivery of new enzymes to phagosomes or by fusion of the latter with preexisting lysosomes. In our studies, the phagosomes (large coated vesicles) clearly fuse with preexisting lysosomes (multivesicular bodies), but, in addition, new enzyme activity is acquired by the lysosomes. In fact, in our system, introduction of protein



FIGURES 37–39 Stacks of Golgi cisternae (*Gc*) from peroxidase-perfused specimens (20 min) incubated with GP. The amount of reaction product present, the number of reactive cisternae, and the number of reactive, coated vesicles (arrows) seen in the vicinity are greater than in controls. Fig. 37, × 70,000; Fig. 38, × 105,000; Fig. 39, × 78,000.

material into the lumen and its subsequent uptake appear to trigger the movement of lytic enzymes from Golgi elements to lysosomes, for increased numbers of small coated vesicles, some of which contain AcPase activity, are seen near multivesicular bodies at 40 min after peroxidase injection. Since the lytic enzymes are carried by small coated vesicles, these structures correspond to primary lysosomes. Previously, Holtzman et al. (36, 37) have reported the presence of AcPase activity in coated vesicles of neurons from the rat ganglion nodosum and have concluded that such vesicles are lysosomes derived from GERL elements.

Coated Vesicles

Specialized vesicles distinguishable by the presence of a highly organized layer of material on the cytoplasmic surface have been described in a variety of tissues as "complex" (38), "bristle-coated" (11), "alveolate" (39), or "dense rimmed" (40). It was Roth and Porter who first proposed that such vesicles may have a specialized function. Based on their findings on uptake of yolk protein by insect oocytes (11, 41) and on incorporation of ferritin-conjugated albumen into hepatocytes (42), they postulated that coated vesicles are specialized for the cellular uptake of protein. Additional work by others on absorption of specific proteins, such as hemoglobin (5, 6, 31), ferritin (9, 41, 43), albumen (8), peroxidase (7, 10), and hemolymph (35), in several tissues (notably kidney and epididymal epithelia) support this hypothesis.

In addition to their association with the apical cell surface and protein uptake, coated vesicles have also been associated with the lateral cell membrane (39, 44), with elements of the ER, and with the Golgi complex. They have been described as occurring frequently in the Golgi region, sometimes in direct continuity with the Golgi cisternae (37, 44, 45). On the basis of such findings it has been postulated (44) that coated vesicles are derived from Golgi elements and that they may be involved in the transport of enzymes (37, 44). It has also been suggested (34) that coated vesicles may transport soluble products of intracellular digestion. Up to the present, however, there has been no direct evidence to support either of these hypotheses, or to indicate the direction of movement of the vesicles, i.e. away from or toward Golgi

cisternae. Our observations provide such evidence by demonstrating that there is a distinct population of coated vesicles, distinguishable by their smaller size, and lytic enzyme content which is derived from the Golgi complex and which functions, in part, in the transport of lytic enzymes to lysosomes. It should be emphasized, however, that in all likelihood the transport functions of coated vesicles are multiple. In this regard, our findings suggest that some of the Golgi-associated coated vesicles fuse with the apical cell membrane and could serve either (*a*) to convey enzymes and/or surface-coat material to the apical plasmalemma, or (*b*) to replace membrane lost from the cell surface during protein absorption.

Our observations also suggest that in the epithelium of the rat vas deferens the bristle coat, persistent or transient, largely characterizes elements of the vacuolar or lysosomal system (14): the apical pinocytic invaginations, heterophagosomes, digestive vacuoles, and primary lysosomes of Golgi origin.

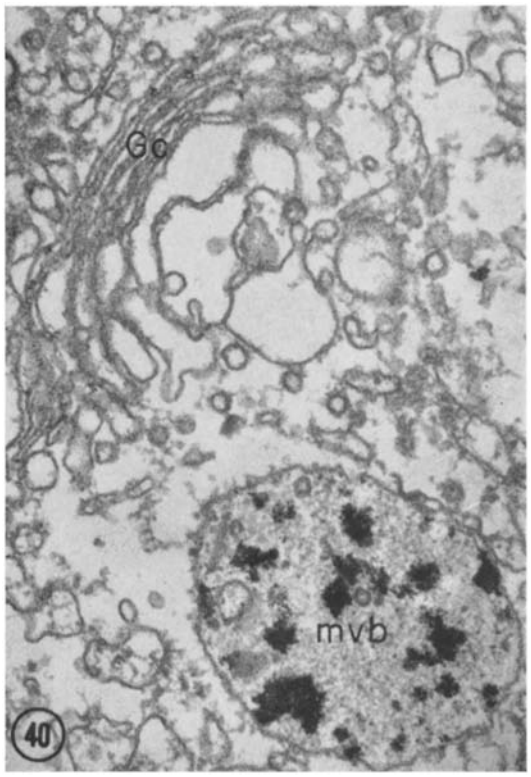


FIGURE 40 Golgi region from a peroxidase-perfused specimen (20 min) incubated with CMP. No reaction product is seen in the Golgi cisternae (*Gc*) and associated vesicles, but dense deposits are present in the multivesicular body (*mvb*) located nearby. × 52,000.

Cytochemical Results

It is of interest that peroxidase was found within the various elements of the vacuolar system (14), but it did not gain access, in concentrations which could be detected by our methods, to other intracellular compartments, such as the ER and Golgi complex. Furthermore, contrary to the findings of Sedar (10), we did not detect peroxidase between cells, except in those areas which had been noticeably damaged by the experimental procedures.

Cytochemical tests with TPP, CMP, and GP were carried out in the hope of identifying the sites of relocation of the small coated vesicles which move away from the Golgi region after peroxidase infusion. Results with TPP were disappointing in that only those coated vesicles in the immediate vicinity of Golgi cisternae or in direct continuity with them were reactive. TPP-positive vesicles were not seen elsewhere in the cytoplasm or in association with other cellular organelles. Hence we conclude that the enzyme activity is usually lost or at least not demonstrable away from the Golgi zone and that this technique is not useful in tracing vesicle relocation in this system.

The results with GP and CMP were more rewarding in that some of the small coated vesicles found near multivesicular bodies in the peripheral cytoplasm as well as those in the Golgi zone, were reactive in both control and peroxidase-infused specimens. Based on results of vesicle counts, we have concluded that their direction of movement is from the Golgi complex to lytic bodies and that the small coated vesicles are primary lysosomes. Attempts to count directly the vesicles in cytochemical preparations were abandoned, owing to the variation in reactivity of different specimens and especially to the difficulty in distinguishing between coated and smooth vesicles after incubation at acid pH. Blurring of the coats in such preparations or tissue differences may explain why, with the exception of recent publications by Holtzman et al. (36, 37), in previous studies (30, 22) no coats have been recognized around AcPase positive vesicles in the Golgi region.

Finally, note should be made of the fact that our results with CMP and with GP were not identical. In both control and peroxidase-perfused

preparations, considerable activity was present along the surface plasmalemma and in intermicrovillar pits with GP but not with CMP. Furthermore, after peroxidase infusion, activity in Golgi cisternae was increased with GP and reduced with CMP. These findings indicate that the enzyme activities demonstrated are not strictly parallel, and that these two substrates cannot be used interchangeably.

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