

The Cytoplasmic Droplet of Rat Epididymal Spermatozoa Contains Saccular Elements with Golgi Characteristics

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Abstract. The cytoplasmic droplet of epididymal spermatozoa is a small localized outpouching of cytoplasm of the tail of unknown significance. EM revealed flattened saccular elements as the near exclusive membranous component of the droplet. Light and electron microscopic immunolabeling for Golgi/TGN markers showed these saccules to be reactive for antibodies to TGN38, protein affinity-purified α 2,6 sialyltransferase, and anti-human β 1,4 galactosyltransferase. The saccules were isolated by subcellular fractionation and antibodies raised against this fraction immunolabeled the saccules of the droplet in situ as well as the Golgi region of somatic epithelial cells lining the epididymis. The isolated droplet fraction was enriched in galactosyltransferase and sialyltransferase

activities, and endogenous glycosylation assays identified the modification of several endogenous glycopeptides. EM lectin staining in situ demonstrated galactose and *N*-acetyl galactosamine constituents in the saccules. Endocytic studies with cationic and anionic ferritin as well as HRP failed to identify the saccules as components of the endocytic apparatus. Epididymal spermatozoa were devoid of markers for the ER as well as the Golgi-associated coatamer protein β -COP. It is therefore unlikely that the saccular elements of the droplet participate in vesicular protein transport. However, the identification of Golgi/TGN glycosylating activities in the saccules may be related to plasma membrane modifications which occur during epididymal sperm maturation.

THE cytoplasmic droplet of epididymal spermatozoa is present in all mammalian species and has remained an enigma since its original discovery by Retzius (1909) with speculations that its membranous components represented degenerating Golgi elements (Bloom and Nicander, 1961) or lysosomes (Dott and Dingle, 1968). The droplet is transiently retained coincident with the maturation of spermatozoa in the epididymis. In the rat caput epididymidis it migrates along the mid-piece of the tail from the neck region of the spermatozoon to the annulus and eventually detaches from sperm in the corpus and cauda epididymidis to be endocytosed by the epithelial clear cells lining these regions of the duct (Hermo et al., 1988; Robaire and Hermo, 1988).

Since the cytoplasmic droplet persists throughout the period of sperm maturation, we sought to identify and isolate the components of this structure and thereby evaluate its derivation. Here we show by morphological analysis, light microscopic (LM)¹ and electron microscopic immunolabeling, subcellular fractionation, exogenous and endogenous glycosylation studies that the cytoplasmic droplet contains loosely organized saccules retaining Golgi/TGN character-

istics. The saccules are not a component of the endocytic apparatus. In the absence of detectable ER markers or the coatamer protein β -COP in epididymal spermatozoa, a role in protein transport is unlikely. However, the saccules may serve to modify the plasma membrane during epididymal sperm maturation.

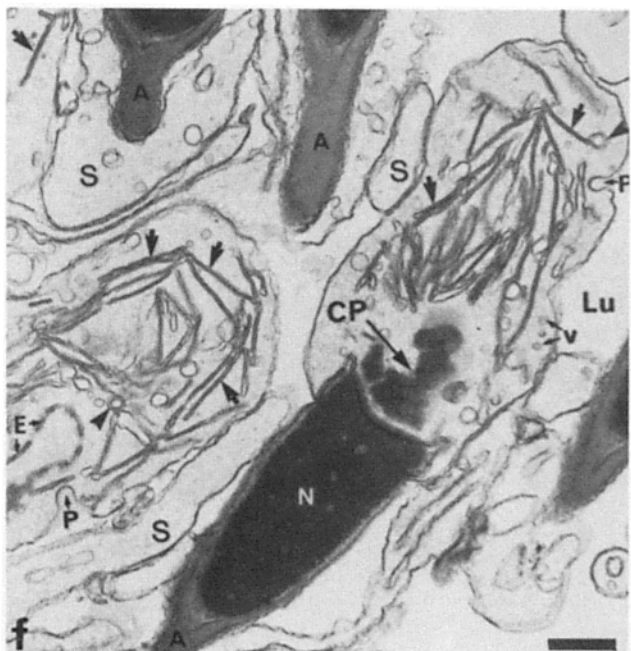
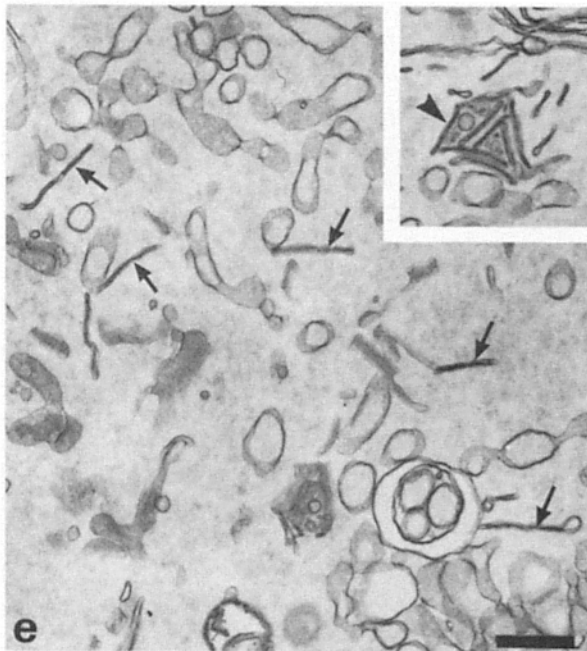
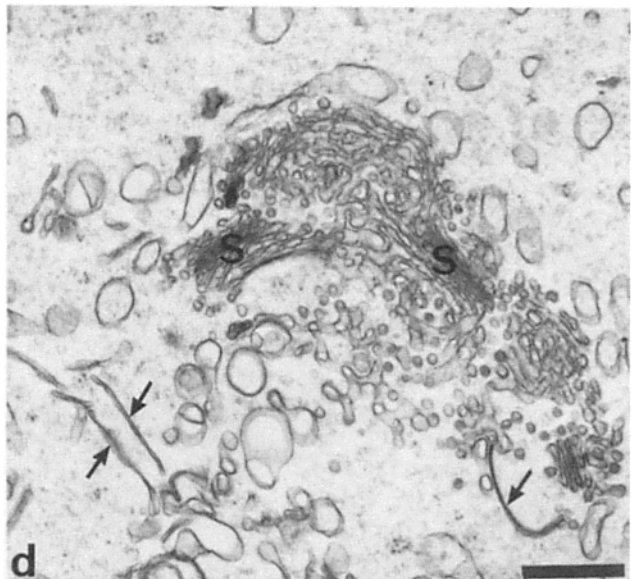
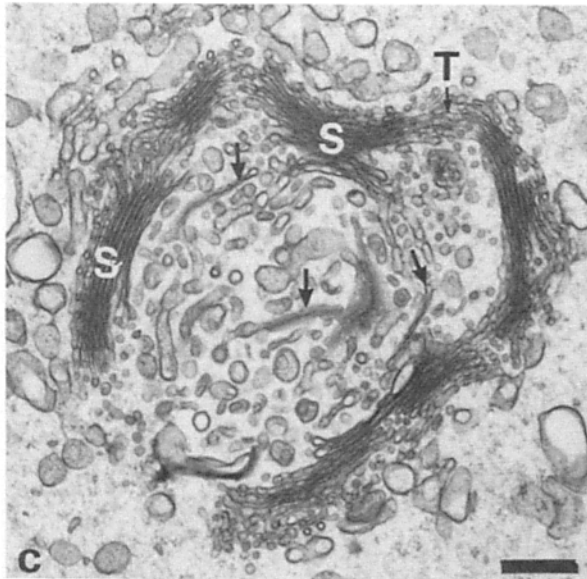
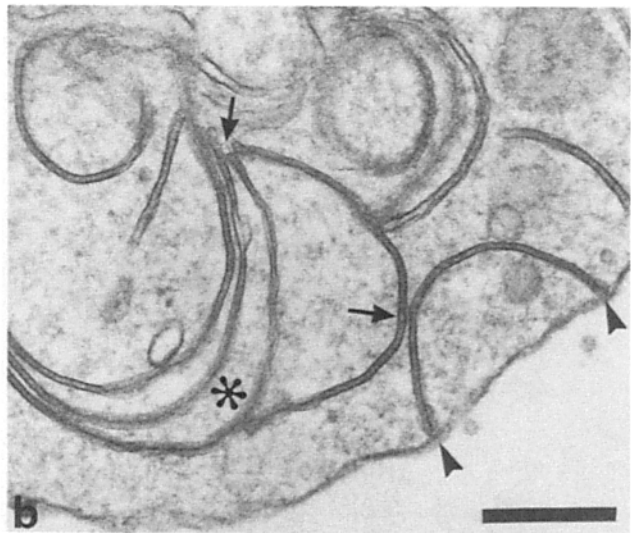
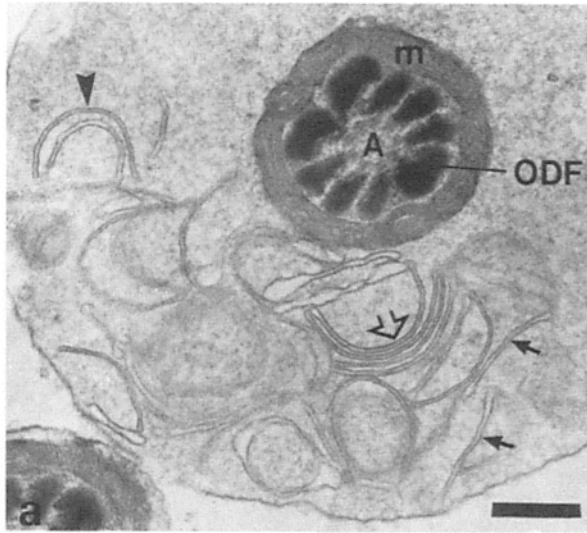
Materials and Methods

Morphology, Light, and Electron Microscope Immunolabeling Procedures

EM analysis of routinely fixed rat testis and epididymis embedded in Epon was as described by Hermo et al. (1991b). LM immunocytochemistry used antibody linked to peroxidase to visualize the primary antibodies via diaminobenzidine polymerization as was described by Oko and Clermont (1989) and Hermo et al. (1991a). EM immunolabeling of thin sections of Lowicryl-embedded epididymis was as described by Oko (1988) and Hermo et al. (1991a) using 10-nm gold-labeled secondary antibodies. Ultrathin frozen sections of subcellular fraction were prepared for EM immunolabeling as described by Duden et al. (1991). Attempts at immunolabeling of frozen thin sections of testis and epididymis were unsuccessful due to the poor morphology of the paraformaldehyde fixed male reproductive tissue. The source of the anti- β 1,4 galactosyltransferase was from Dr. E. Berger (Physiologisches Institut, Zurich, Switzerland) and was antiserum N₇ raised to native human milk galactosyltransferase (Childs et al., 1986). The anti 2,6 sialyltransferase was from Dr. J. Paulson (Cytel Corp., San Diego, CA) and was IgG affinity purified from bacterially expressed sialyltransferase (Taates et al., 1988). The anti-TGN38 was antiserum obtained from Dr. K. Howell (University of Colorado, Denver, CO) and has been described and characterized in Luzio et al. (1990). The antibody to β -COP was polyclonal

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1. *Abbreviations used in this paper:* CMP, cytidine 5' monophosphate; HPL, Helix Pomatia lectin; LM, light microscopic; RCA I, Ricinus Communis Agglutinin I.



antisera raised to a peptide comprising amino acids 496–513 of β -COP (anti-EAGE) as characterized by Duden et al. (1991). Lectin binding studies using Ricinus Communis Agglutinin I (RCA I) and Helix Pomatia lectin (HPL) linked to 15 nm colloidal gold was as described by Hermo et al. (1992). Tracer studies were performed as described by Hermo and Morales (1984). Cationic ferritin (adsorptive marker) and anionic ferritin and HRP conjugated to colloidal gold (fluid-phase markers) were injected into the lumen of the rete testis and at various time intervals thereafter, i.e., from 2 min to 24 h, the animals were sacrificed and fixed by perfusion with 2.5% glutaraldehyde buffered in sodium cacodylate (0.1 M). The tissue was then processed for routine EM analysis as described by Hermo et al. (1991b).

Subcellular Fractionation, Exogenous, and Endogenous Glycosylation and Acid Phosphatase Assays

Rat initial segment and caput epididymides perfused in vivo with saline were dissected out and cut with a razor blade (all procedures were carried out at 4°C) into 1–2-mm cubes in a buffer of 0.25 M sucrose, 50 mM Tris-HCl, pH 7.4, 25 mM KCl, 1 mM PMSF and 100 U/ml of aprotinin. By gentle mixing, spermatozoa were observed to exude as a milky suspension from the tissue cubes. After filtration successfully through a coarse metal mesh (Fisher Scientific Co., Montreal, Quebec) followed by a 150- μ M Nitex net (Thompson, Montreal, Quebec), the exudate was observed to contain mainly spermatozoa as evaluated by phase contrast and EM. After washing the sperm suspension (15 min, 1,500 g), the pellet, which contained cytoplasmic droplets attached or unattached to spermatozoa, was resuspended in the above buffer. The supernatant which also contained detached cytoplasmic droplets was pelleted (150,000 g for 15 min) and subsequently resuspended together with the other pellet. The pooled suspension was homogenized by passage (five times) through a 20-gauge needle which assured complete detachment of all cytoplasmic droplets as evaluated by phase-contrast microscopy. The homogenate was centrifuged (150 g for 10 min) and the supernatant enriched in droplets was loaded and layered over a discontinuous gradient of 0.6, 0.8, 1.0, 1.2 M sucrose in the above buffer. After centrifugation, (200,000 g_{av} for 1.5 h), fractions were collected from each of the interfaces and along with the pellet fraction and the remainder of the gradient saved for enzyme assays and protein content. Fraction 3 was also used in the production of rabbit polyclonal antibodies in rabbits by a similar protocol as described by Oko (1988). The antibodies detected three major proteins of the cytoplasmic droplet fraction of 14.5, 23, and 41 kD as assayed by immunoblot analysis and will be described in detail elsewhere.

Exogenous glycosylation assays for galactosyltransferase with ovomucoid as acceptor and sialyltransferase with asialofetuin as acceptor were as described by Bergeron et al. (1985). Endogenous glycosylation whereby the transfer of galactose from UDP- 3 H]galactose at pH 6.5 to endogenous acceptors or of sialic acid at pH 5.8 from cytidine 5' monophosphate (CMP)- 3 H]sialic acid to endogenous acceptors of Golgi fractions was carried out exactly as described for the optimal conditions in Bergeron et al. (1985) with the additional proteinase inhibitors: aprotinin, 200 U/ml; leupeptin, 10 μ g/ml; pepstatin, 1 μ g/ml; pepabloc, 1 mM (Boehringer Mannheim, Montreal). Acid phosphatase reactivity was assayed as described in Bergeron et

al. (1982) and protein content was determined by the method of Bradford (1976).

Results

Morphology

EM revealed that the cytoplasmic droplet of epididymal spermatozoa consisted of an array of flattened elements localized to one pole of the droplet (Fig. 1, *a* and *b*). Grazing sections (Hermo et al., 1988) confirmed the saccular nature of these structures (as opposed to tubules) which were occasionally in close apposition to each other as well as to the plasma membrane (Fig. 1 *b*). Prominent among possibilities for the derivation of the saccular structures was the Golgi apparatus of precursor late spermatids of the testis. In early spermatids (steps 1–7) the Golgi apparatus was hemispherical, and occupied the cranial pole of the nucleus, where an involvement in acrosome formation has been demonstrated (reviewed in Clermont et al., 1990). At step 8, the Golgi apparatus was observed at the caudal pole of the nucleus next to the developing tail with a spherical morphology and thereafter was free in the cytoplasmic lobe of the spermatid. Morphological analysis of steps 9–15 spermatids revealed a classically oriented Golgi apparatus with stacked saccules and associated vesicles (Fig. 1 *c*). By step 16, the Golgi apparatus appeared less intact with dispersed saccules now evident (Fig. 1 *d*). At step 17 of spermiogenesis, the Golgi apparatus was no longer observed as a morphological structure. However, many flattened saccular elements were observed either freely distributed in the surrounding cytoplasm (Fig. 1 *e*) or in loose aggregates (*inset*). Morphological analysis of step 19 spermatids revealed a segregation of unused mitochondria, granulated bodies, endoplasmic reticulum, ribosomes and other organelles into the residual body destined for phagocytosis by Sertoli cells (Morales et al., 1985). The flattened saccules, on the other hand, congregated within the small mass of leftover cytoplasm in the neck region of the tail which by late step 19 spermatids was recognizable as the cytoplasmic droplet (Fig. 1 *f*).

LM and EM Immunolabeling with Golgi Apparatus Markers and Lectin Staining In Situ

Detection of Golgi markers in situ was attempted by immunocytochemistry (Fig. 2). Antibodies to β 1,4 galactosyl-

Figure 1. Golgi apparatus rearrangement during sperm development. (*a*) Cross section through the tail of a spermatozoon at the level of the cytoplasmic droplet in the lumen of the initial segment of the epididymis showing loosely stacked (*open arrow*), straight (*arrows*), or C-shaped (*arrowhead*) flattened saccular elements. *m*, mitochondrial sheath; *ODF*, outer dense fiber; *A*, axoneme. (*b*) At higher power the flattened saccular elements show areas of close apposition (*arrows*) but large gaps between elements are also observed (*asterisk*). The edges of the saccular elements are frequently observed in close proximity to the plasma membrane (*arrowheads*). (*c*) Golgi apparatus of a step 10 spermatid showing stacks of Golgi saccules (*S*) with tubular connections (*T*) in the large spherical Golgi apparatus. On the trans face, tubulo-vesicular profiles of different sizes are present including what appear to be detached saccular elements (*arrows*). (*d*) Cytoplasm of a step 16 spermatid. Flattened saccular elements (*arrows*) are seen at a distance from the stacks of Golgi saccules (*S*) which are far less elaborate and conspicuous. The Golgi apparatus is no longer spherical. (*e*) Cytoplasm of a step 18 spermatid. The inset at the upper right corner shows a small remnant of what may be the Golgi apparatus containing saccular elements (*arrowhead*). Randomly distributed throughout the cytoplasm are many flattened saccular elements (*arrows*). (*f*) Late step 19 spermatids in the lumen (*Lu*) of the seminiferous tubule at stage VIII of the cycle of the seminiferous epithelium prior to spermiation. These spermatids are still closely associated with cytoplasmic processes of Sertoli cells (*S*). Cytoplasmic droplets of these spermatids appear at the level of the connecting piece (*CP*) or neck region of their tails. The droplets contain many flattened saccular elements (*arrows*) which are loosely arranged or closely stacked; they contain only the occasional small vesicular profile (*v*) and a few spherical bodies with a double membrane (*arrowheads*). The plasma membrane in the region of the droplet shows several invaginations or pits (*P*). *N* and *A*, nucleus and acrosomic system of the spermatid head, *E*, nuclear envelope. Bars: (*a* and *b*) 0.3 μ m; (*c–f*) 0.5 μ m.

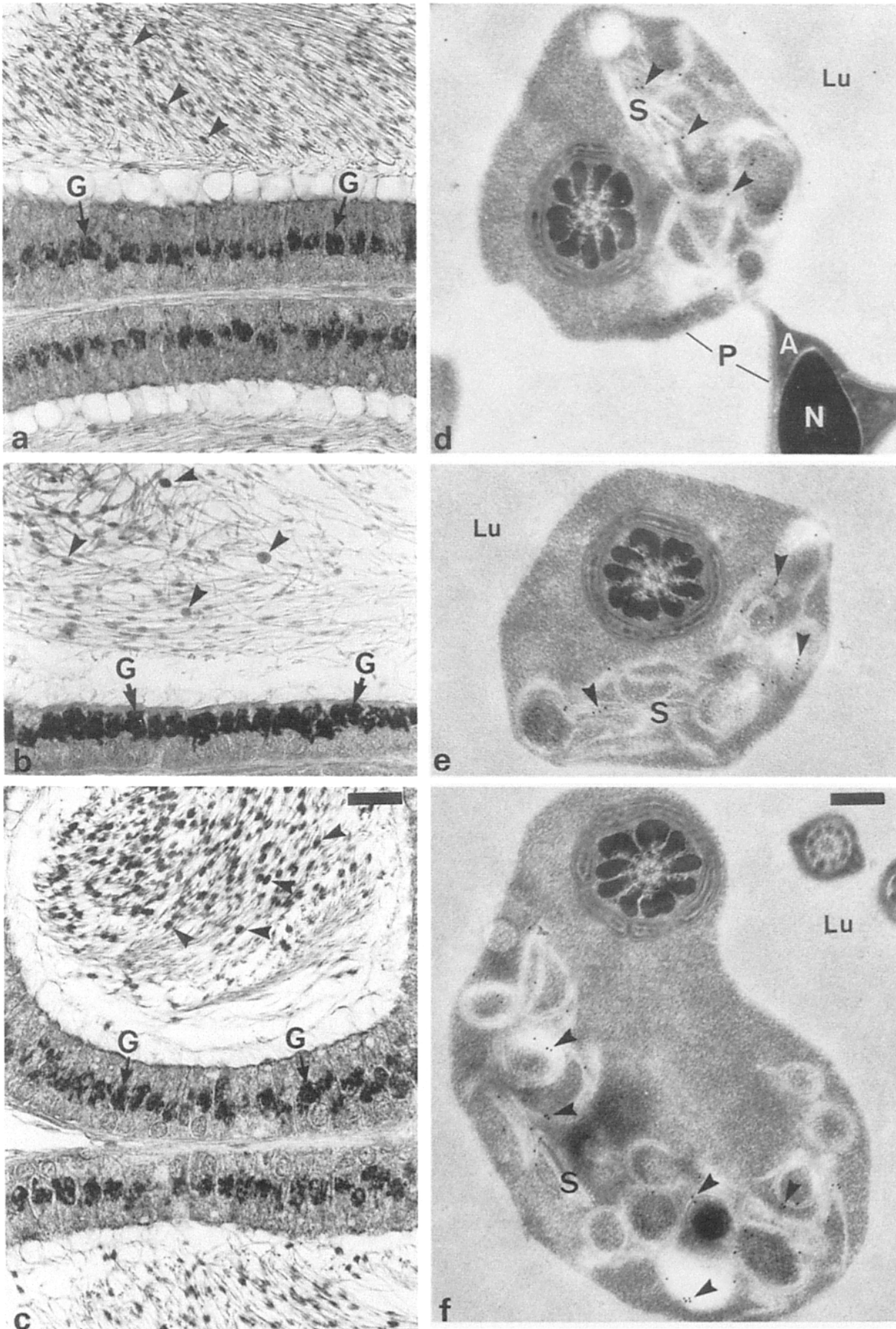


Figure 2. Localization of Golgi markers in the cytoplasmic droplet. Light micrograph of the initial segment and caput epididymidis immunostained with (a) anti- β 1,4 galactosyltransferase (1:50); (b) anti- α 2,6 sialyltransferase (1:10); (c) anti-TGN38 (1:20) revealing reactivity to the Golgi apparatus (G) of the epithelial principal cells and cytoplasmic droplets (arrowheads) of spermatozoa in the lumen while the tails of the spermatozoa are unreactive. (d, e, and f) EM immunolocalization of 10-nm gold-decorated secondary antibodies to primary

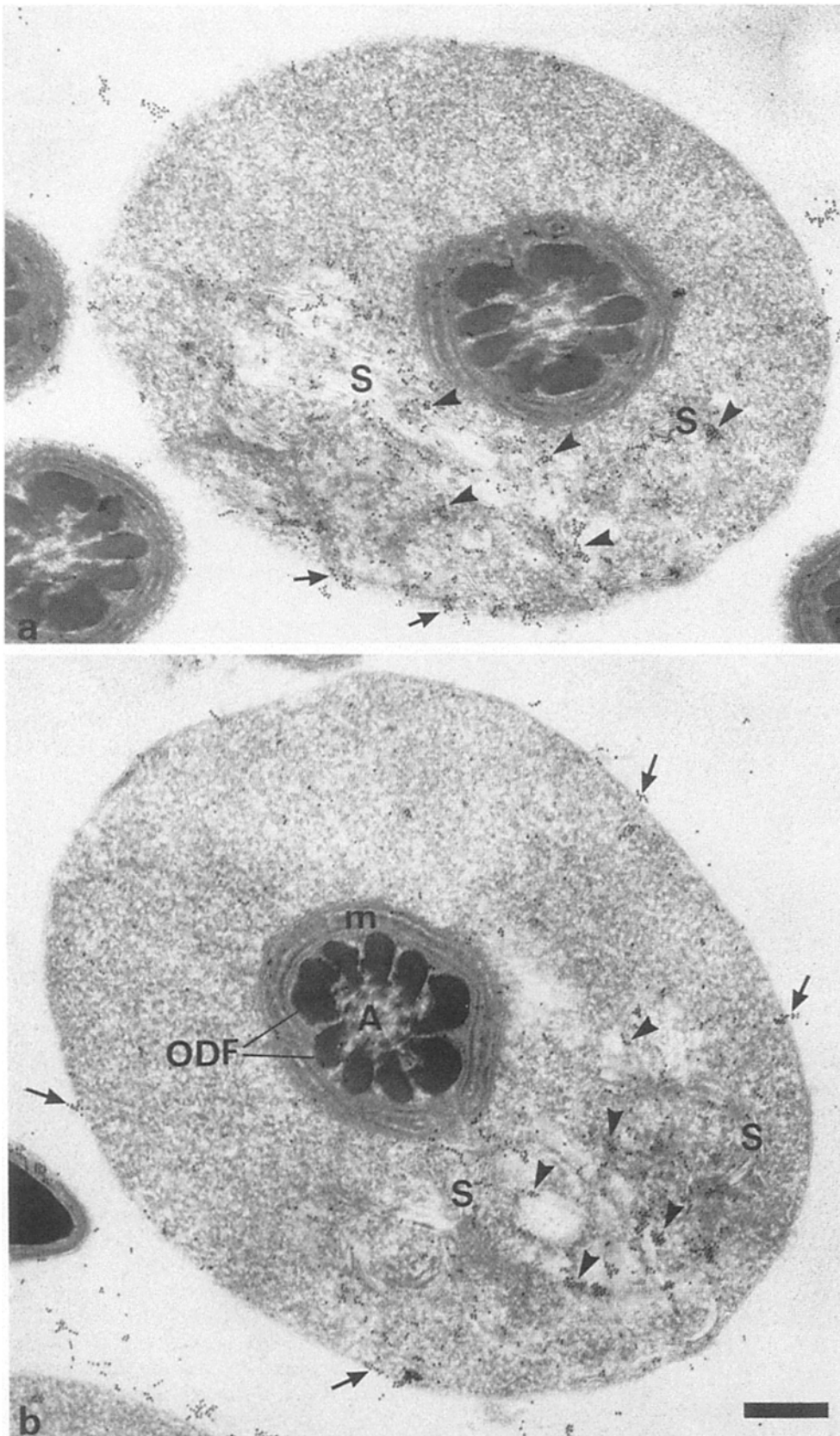


Figure 3. Lectin labeling of cytoplasmic droplets within the caput epididymidis. (a) RCA-I colloidal gold (15 nm) decorated section of the droplet showing numerous gold particles (*arrowheads*) over the saccular elements (*S*) occupying one pole of the droplet indicating the presence of D-galactose containing glycoconjugates. Label is also seen over the plasma membrane (*arrows*). (b) Staining with HPL colloidal gold complexes. The majority of gold particles (*arrowheads*) are present over the saccular elements (*S*) indicating the presence of N-acetyl galactosamine containing glycoconjugates. Gold particles are also seen over the plasma membrane (*arrows*). *A*, axo-neme; *ODF*, outer dense fibers; *m*, mitochondrial sheath. Bar, 0.3 μm .

antibodies against β 1,4 galactosyltransferase (1:50), α 2,6 sialyltransferase (1:5) and TGN38 (1:2), respectively, within droplets of the caput epididymidis. In all cases, gold particles (*arrowheads*) are situated over the saccules. There is no labeling over the plasma membrane (*P*) of the droplet or sperm head. The acrosome (*A*) and nucleus (*N*) of the sperm head are indicated as well as the lumen (*Lu*) of the epididymis. Bars: (a-c) 20 μm ; (d-f) 0.3 μm .

Table I. Distribution of Golgi and Lysosomal Marker Enzymes

	Fraction P		Fraction S	
	Percent*	RSA‡	Percent	RSA
Protein	74.2 ± 3.5		25.3 ± 3.5	
Galactosyltransferase	58.7 ± 5.9	0.79 ± .07	41.3 ± 5.9	1.65 ± 0.25
Sialyltransferase	57.5 ± 7.7	0.78 ± .02	42.5 ± 7.7	1.68 ± 0.55
Acid phosphatase	N.D.		100	3.9 ± 0.5

Spermatozoa of the initial segment and caput epididymidis fractionated into P and S fractions. The enzyme activities for the S fraction were as follows: Galactosyltransferase, $0.19 \pm .03$ nmols galactose transferred · min/mg protein; Sialyltransferase, $0.114 \pm .02$ nmols sialic acid transferred · min/mg protein; Acid phosphatase, 68 ± 11.6 nmols PNPP hydrolyzed · min/mg protein.

* Percent of total recovered protein or enzyme activities (mean ± SD, $n = 3$).

‡ Calculated as the percent recovered enzyme activity/percent recovered protein (mean ± SD, $n = 3$).
N.D., not detectable.

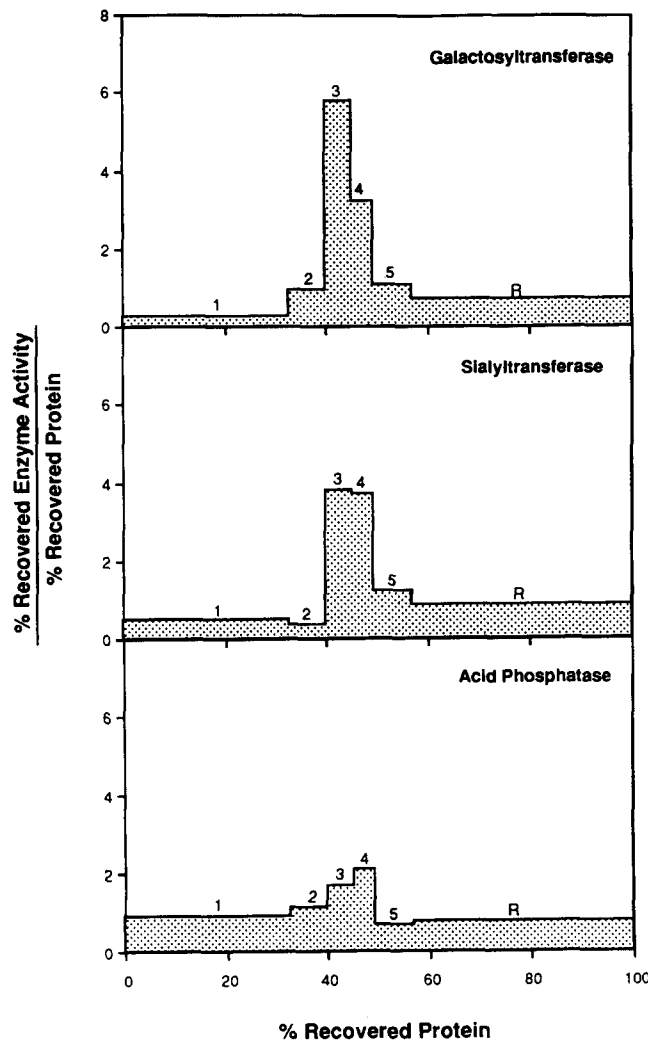


Figure 4. Isolation of the cytoplasmic droplet. The supernatant fraction isolated as described in Table I was overlaid on a discontinuous gradient of 0.6, 0.8, 1.0, 1.2 M sucrose in 50 mM Tris HCl, pH 7.4, 25 mM KCl with aprotinin and PMSF. Each interface of the gradient from bottom to top of the tube was collected (fractions 1-5 with fraction 1 the resuspended pellet) with the remainder of the material collected and designated fraction R. Aliquots of the fractions were then evaluated for the indicated enzyme activities and protein content. The results are presented as a modified de

transferase, $\alpha 2,6$ sialyltransferase, and TGN38 clearly demarcated the Golgi region of the somatic epithelial principal cells of the epididymis (Fig. 2, a-c). Moreover, in the LM the droplets of spermatozoa of at least the initial segment and caput epididymidis were also specifically immunostained (Fig. 2, a-c); non-immune serum showed no reactivity (not shown). EM immunogold labeling of Lowicryl sections revealed gold particles exclusively over the saccular elements of the cytoplasmic droplet of spermatozoa of the initial segment and caput epididymidis with no labeling of the plasma membrane observed (Fig. 2, d-f). EM immunolabeling with all three antibodies was also observed exclusively on the Golgi apparatus and associated TGN of the entire epididymal epithelial principal cells, whereas controls with non-immune serum showed no immunoreactivity of droplets or principal cells (not shown). Incubation of Lowicryl sections with HPL and RCA I lectin-gold complexes displayed binding to the saccular elements of the cytoplasmic droplet as well as the adjacent plasma membrane of initial segment and caput epididymal sperm (Fig. 3, a and b), whereas the corresponding lectin in the presence of its competitive sugar showed no labeling by lectin gold (not shown).

Isolation and Characterization of the Saccular Elements within the Cytoplasmic Droplet

The distribution of Golgi apparatus and lysosomal markers was evaluated in subcellular fractions obtained from homogenates of the initial segment and caput epididymal spermatozoa. Isolation of a low speed supernatant revealed enrichment for these markers (Table I). Further fractionation by discontinuous gradient centrifugation revealed a 9.6-fold enrichment in homogenate galactosyltransferase activity in fraction 3 which also revealed a 6.4-fold enrichment in sialyltransferase (Fig. 4). Acid phosphatase was less prominent with the highest enrichment found in fraction 4. EM analysis of fraction 3 revealed profiles of cytoplasmic droplets showing saccular elements enclosed within the remnant plasma membrane (Fig. 5 a). Incubation of frozen sections of this fraction at the EM level revealed strong im-

Duve plot describing the mean ($n = 3$) of three fractionations with relative enrichments of enzyme activity in the fractions of the gradients plotted as a function of the relative protein content recovered for each fraction of the gradient.

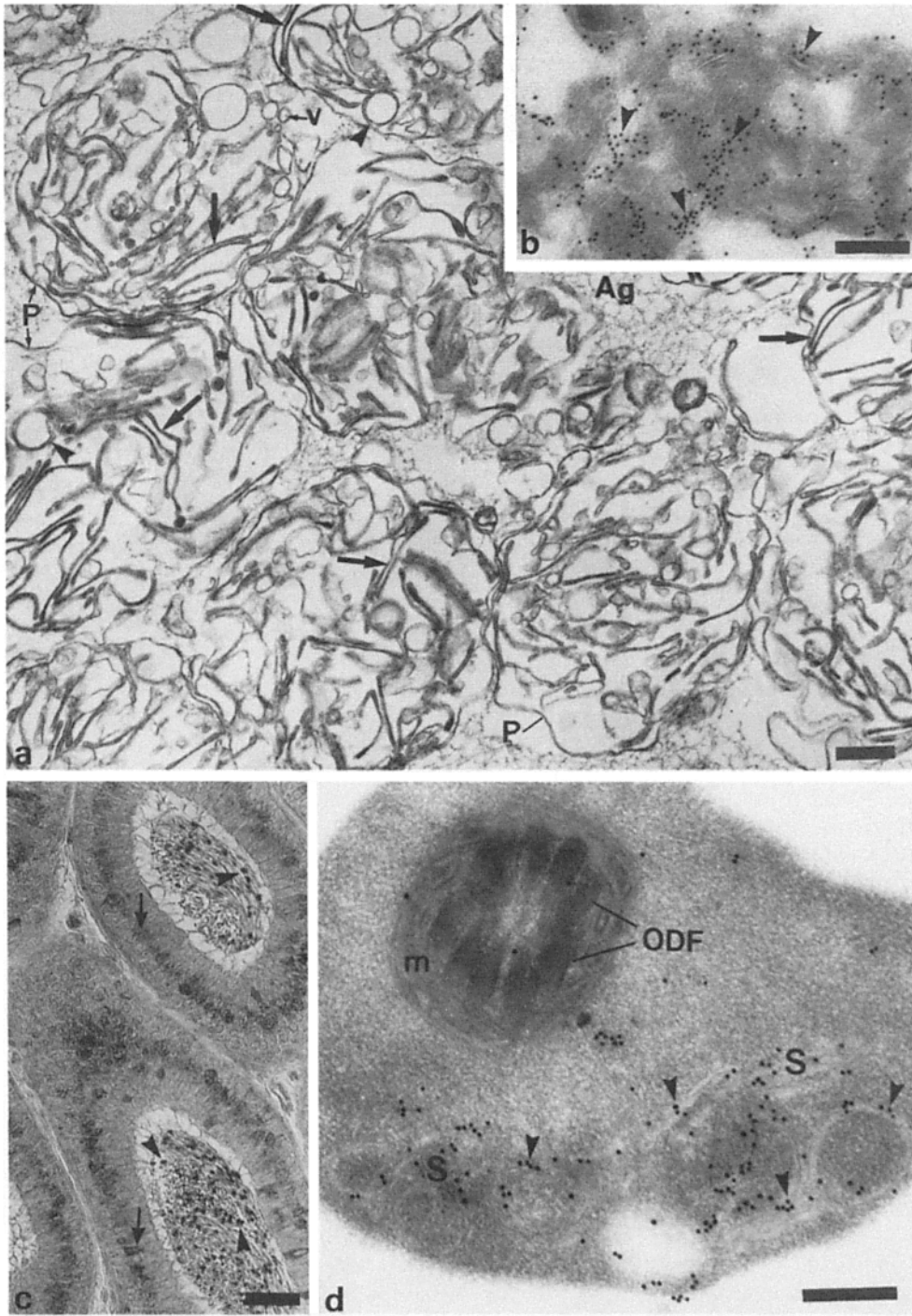


Figure 5. Morphological analysis of the isolated droplet and its immunogenic localization. (a) EM of fraction 3 showing numerous flattened straight or C-shaped saccular elements (arrows), enclosed within a remnant plasma membrane (P). Ag, agarose embedding material; arrowheads, spherical bodies with a double membrane; v, vesicular profiles. (b) EM of a frozen section obtained from fraction 3 immunolabeled with anti- β 1,4 galactosyltransferase showing abundant gold particles (arrowheads) located over pale saccular elements. (c) LM of several tubules of the initial segment of the epididymis immunostained with an antibody raised against fraction 3. Immunoperoxidase reaction product is present over the cytoplasmic droplets of spermatozoa (arrowheads) in the lumen as well as the Golgi apparatus (arrows) of the epithelial principal cells. (d) EM of a cytoplasmic droplet in situ immunolabeled with an antibody raised against the isolated fraction 3. Gold particles (arrowheads) decorate the saccular elements (S) occupying one pole of the droplet. ODF, outer dense fibers; m, mitochondrial sheath. Bars: (a, b, and d) 0.3 μ m; (c) 40 μ m.

munoreactivity to anti-galactosyltransferase (Fig. 5 b), although the morphology of the fraction was compromised by the cryo-fixation protocol. When antibodies were raised to fraction 3, LM immunocytochemistry revealed reactivity exclusively over the cytoplasmic droplets of spermatozoa as well as the Golgi region of the epididymal epithelial principal cells (Fig. 5 c). At the EM level, immunogold labeling was found over the saccular elements of the cytoplasmic droplet of spermatozoa with little label over the plasma membrane (Fig. 5 d).

Endogenous Glycosylation

To define further characteristics of the saccular elements in the cytoplasmic droplet, *in vitro* glycosylation of endogenous substrates was assessed. Incubation of the intact fraction 3 with UDP- 3 H]galactose or CMP- 3 H]sialic acid in the absence of detergent was performed as described previously for rat liver Golgi fractions (Bergeron et al., 1982, 1985). The results (Fig. 6) revealed that polypeptides of molecular masses 62, 34.5, 20.4, and 14.4 kD were labeled pre-

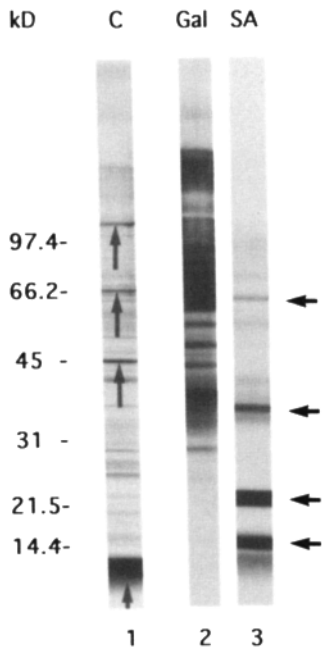


Figure 6. SDS-PAGE of polypeptides of isolated cytoplasmic droplets (lane 1) and endogenous substrates for glycosylation with UDP- ^3H galactose (lane 2) and CMP- ^3H sialic acid (lane 3). Major polypeptides stained by Coomassie brilliant blue (C) were observed at 12, 44.5, 68, and 110 kD (upward pointing arrows). The major sialoprotein acceptors (SA) were observed at 62, 34.5, 20.4, and 14.4 kD (horizontal arrows). Galactose-labeled polypeptides (Gal) were observed between 29 and 280 kD. Molecular mass markers are indicated on the left. $\sim 100 \mu\text{g}$ protein was loaded on lanes 1-3. The exposure time for the x-ray film (lanes 2 and 3) was 1 mo.

dominantly by ^3H CMP sialic acid while a large number of polypeptides of molecular masses between 29 and 280 kD were acceptors for glycosylation with UDP- ^3H galactose (Fig. 7). The sialolabeled glycoproteins did not correspond to major Coomassie blue-stained bands of the fraction which were observed at 12, 44.5, 68, and 110 kD (Fig. 6).

The following experiments were carried out to evaluate if the saccular elements in the cytoplasmic droplet were a component of the endocytic apparatus. Cationic ferritin, anionic ferritin, or HRP bound to colloidal gold were administered into the lumen of the rete testis for time periods of 2 min to 24 h. Although all tracers accumulated in the endocytic components of the somatic epithelial cells lining the epididymis, no detectable tracer was observed in any of the saccular elements of the cytoplasmic droplet at any time after injection. For the specific case of cationic ferritin, the tracer was demonstrated at the cell surface and throughout the endocytic apparatus of lining epithelial cells (Fig. 7, *b* and *c*) but not within the Golgi apparatus/TGN of these somatic cells. Cationic ferritin also clearly labeled the spermatozoan plasma membrane, but no detectable tracer was found in the saccules of the cytoplasmic droplet (Fig. 7 *a*).

The above characteristics including the demonstration of a Coomassie blue-stained polypeptide of 110 kD observed for the cytoplasmic droplet prompted an evaluation of whether or not the Golgi associated coat protein β -COP was present. LM immunocytochemistry revealed β -COP immunoreactivity (polyclonal antibody to a peptide comprising residues 496-513 of β -COP anti-EAGE) (Duden et al., 1991) in the Golgi region of the entire epididymal epithelial principal cells but not detectable in the cytoplasmic droplets of luminal spermatozoa (Fig. 8 *c*). Spermatids of the testis, however, clearly showed immunoreactivity in the cup-shaped Golgi apparatus at the cranial pole of the nucleus of "early" steps 1-7 spermatids (Fig. 8, *a* and *b*) and in the spherical Golgi apparatus of steps 8-16 spermatids. However, no detectable staining was observed in steps 17-19

spermatids. Remarkably, within the testis, the Sertoli cells with a well developed Golgi apparatus (Rambourg et al., 1979) did not reveal detectable anti β -COP immunoreactivity. Controls with non-immune sera showed no reactivity in the testis or epididymis (not shown).

The final markers evaluated were antiserum to the ER membrane protein calnexin (Wada et al., 1991) and anti-ER antiserum (Louvard et al., 1982). Once again, no detectable immunoreactivity was found in the cytoplasmic droplets of spermatozoa in the lumen, although prominent immunoreactivity throughout the cytoplasm of the epithelial principal cells of the entire epididymis was noted (Fig. 9 *b*). By contrast, in the testis, strong staining of spermatogonia, spermatocytes, and spermatids at all steps of spermiogenesis was observed, but cytoplasmic droplets of step 19 spermatids were unreactive (Fig. 9 *a*).

Discussion

The marker enzyme hypothesis has defined organelles on the basis of the sedimentation properties of enzyme activities (de Duve, 1975). Superimposed upon this hypothesis is organelle definition on the basis of the ultrastructural features of a given compartment (Palade, 1975). The recent identification of cytosolic constituents which associate with intracellular membranes to regulate membrane traffic, e.g., the products of the *ras* supergene family (reviewed in Balch, 1990; Hall, 1993; Lippincott-Schwartz, 1993), the coat proteins designated as coatamers (e.g., Rothman and Orci, 1992; Duden et al., 1992) and adapter proteins of clathrin coats (Keen, 1990) also serve to define functional subcompartments of the endocytic and exocytic pathways.

Here, we have attempted to use morphology, the subcellular distribution of predicted marker enzymes in subcellular fractions and immunocytochemistry to elucidate the nature of the saccular elements in the cytoplasmic droplet of epididymal spermatozoa. By morphology, a coincident loss of Golgi stack organization and segregation of saccular elements to the cytoplasmic droplet was observed during spermiogenesis (Fig. 10). By subcellular fractionation, the isolated droplet fraction was enriched in Golgi marker enzyme activities for galactosyltransferase and sialyltransferase to defined exogenous glycoproteins. Immunolocalization with a $\beta 1,4$ galactosyltransferase antiserum demonstrated that the saccules of the droplet, the Golgi region of epithelial principal epididymal cells and the isolated saccular elements were immunoreactive with the antibody. However, this antibody has been reported to be human specific as well as containing antibodies to sugar residues (Childs et al., 1986; Watzele et al., 1991). For this reason we also used antibodies to protein affinity-purified anti- $\alpha 2,6$ sialyltransferase and antisera to TGN38 with essentially the same observation.

To evaluate a contribution from the endocytic/lysosomal system we evaluated the content of acid phosphatase which was low in the isolated droplet fraction. Taken together with past studies of *in situ* EM cytochemistry which failed to reveal any reactivity for acid phosphatase in the cytoplasmic droplet (Herms et al., 1988), we therefore consider it highly unlikely that the droplet represents a modified lysosome, a view proposed by Dott and Dingle (1968). To evaluate whether the saccules of the droplet in part or in whole repre-

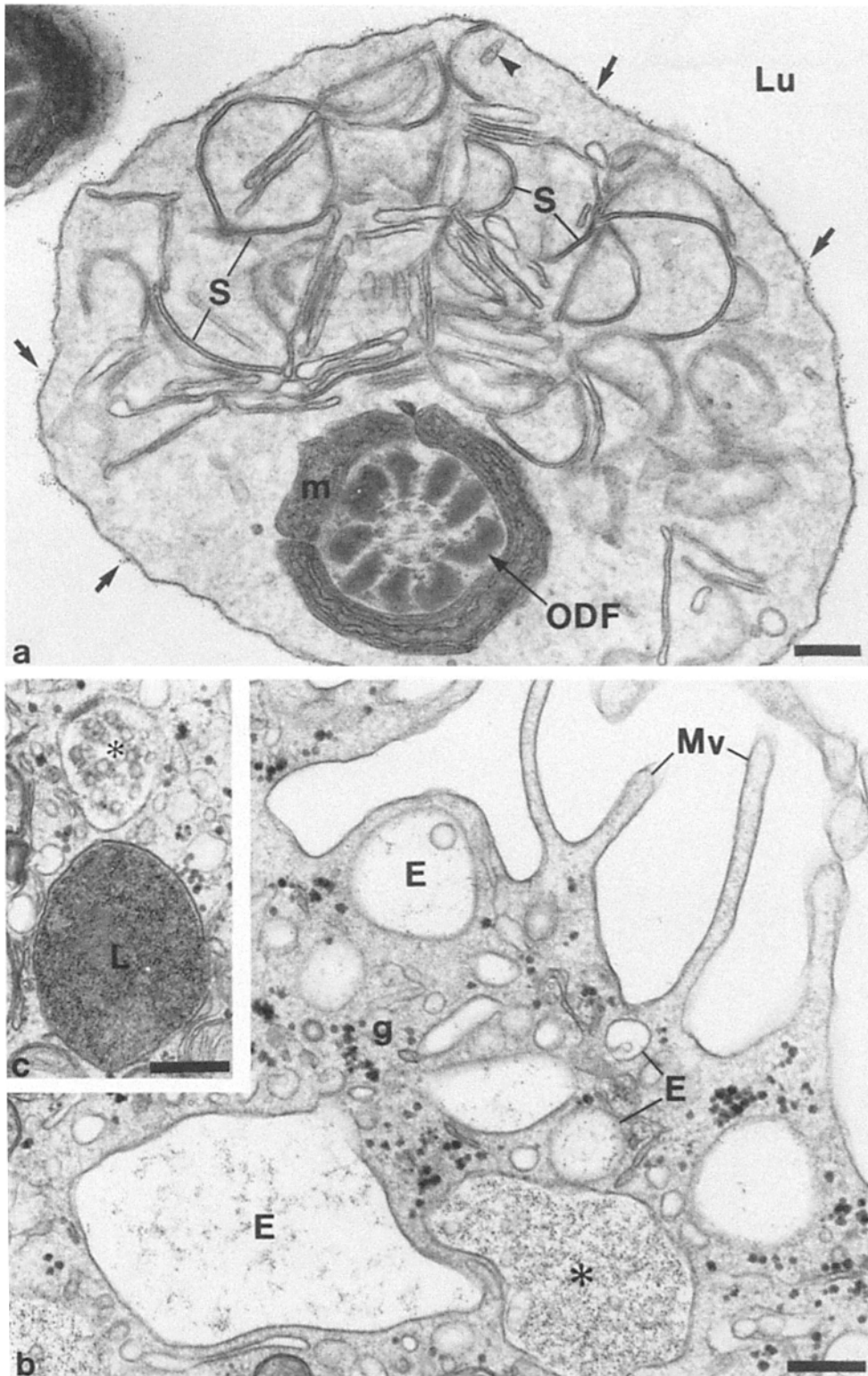


Figure 7. Tracer studies of cationic ferritin injected into the epididymal lumen. (a) Electron micrograph showing an even distribution of cationic ferritin (arrows) on the surface of a cytoplasmic droplet of a spermatozoon within the lumen (Lu) of the efferent duct. The tracer was introduced into the lumen 2 h before fixation. No tracer appears in the lumen of the saccular elements (S) of the droplet. A tubular element is labeled (arrowhead). ODF, outer dense fiber; m, mitochondrial sheath. (b) Electron micrograph of an epithelial cell of the initial segment of the epididymis. 4 h after injection of cationic ferritin into the epididymal lumen, the tracer is present within "early" and "late" endosomes (E) and a multivesicular body (asterisk). g, glycogen; Mv, microvilli. (c) A lysosome (L) and multivesicular body (asterisk) within the same epithelial cell but in the supranuclear region also contain tracer 4 h after its injection into the lumen. Bars, 0.3 μm .

sented other components of the endocytic apparatus, a variety of exogenous tracers (for fluid phase or adsorptive endocytosis) was provided to spermatozoa in the epididymal lumen. No endocytosis within the saccular elements of the droplet was observed even for those tracers (e.g., cationic ferritin) clearly adsorbed to the spermatozoan cell surface,

although a small amount was observed in rare tubular components within the droplet.

A tentative identification of the saccules of the droplet as Golgi derived was supported by the endogenous glycosylation assays which revealed several endogenous substrates for galactosylation and sialylation. While it was not possible to

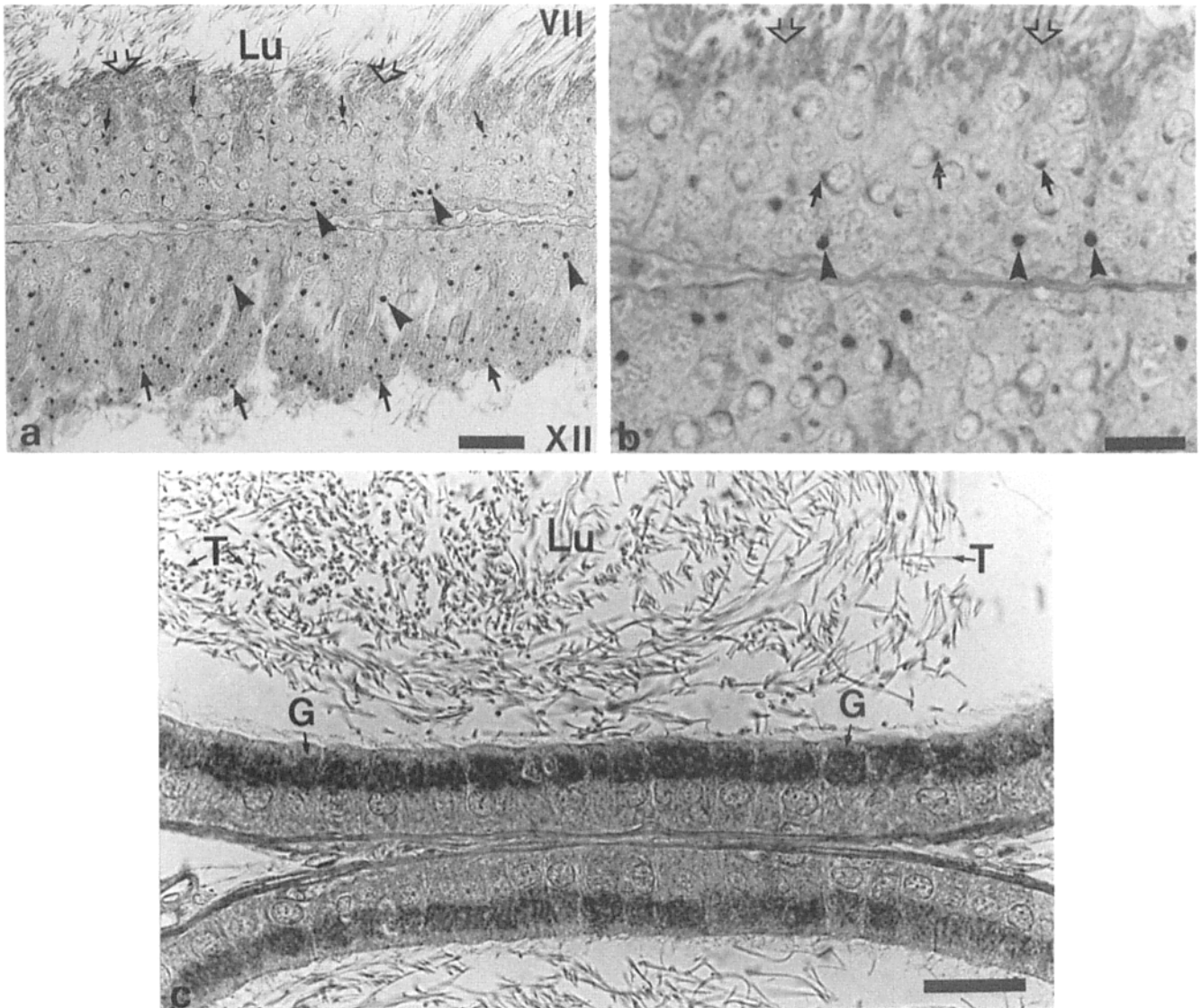


Figure 8.

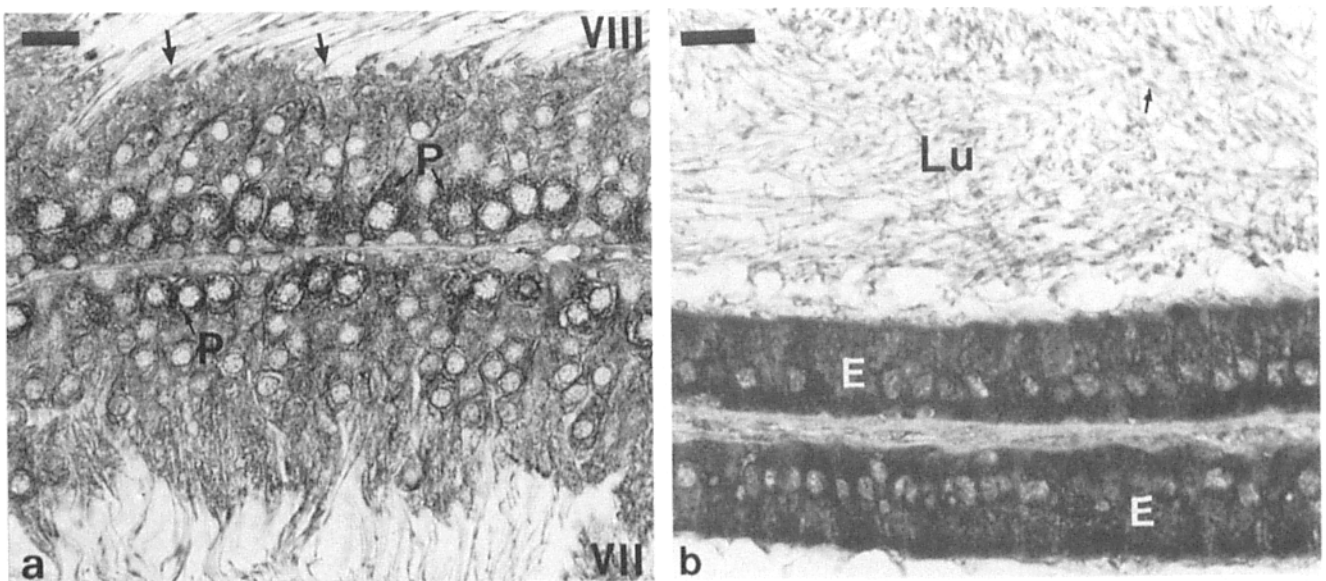


Figure 9.

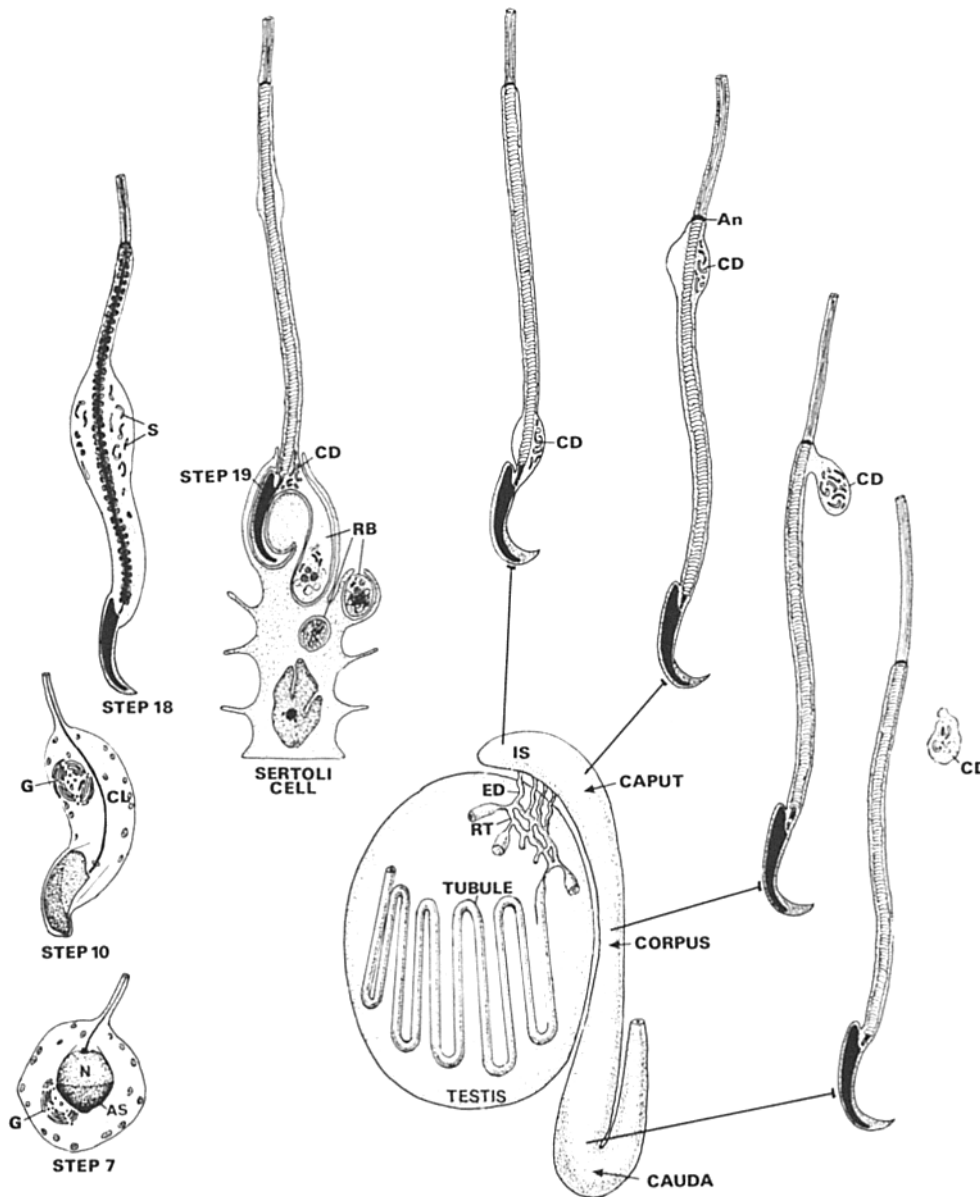


Figure 10. Diagrammatic representation illustrating the Golgi apparatus of early spermatids, its fate in late spermatids and presence in cytoplasmic droplets. In early steps of spermiogenesis (steps 1-8), the Golgi apparatus (*G*) is hemispherical, formed of stacks of saccules and is found next to the acrosomic system (*AS*) overlying the nucleus (*N*). In steps 9-16, the Golgi apparatus becomes spherical and occupies the cytoplasmic lobe (*CL*) of the spermatid. In steps 17-18, the Golgi apparatus is no longer visible as a discrete entity, but many randomly dispersed saccular elements (*S*) occupy the cytoplasmic lobe. At step 19 of spermiogenesis, the saccular elements segregate themselves at the site of the cytoplasmic droplet (*CD*), while the other organelles (unused mitochondria, ER, lipid etc.) collectively become aggregated within the residual body (*RB*). The latter is eventually phagocytosed by the Sertoli cell where it is degraded. The cytoplasmic droplets of spermatozoa in the seminiferous tubules of the testis, rete testis (*RT*), efferent ducts (*ED*), and initial segment (*IS*) are positioned at the level of the neck piece of the tail. In the caput epididymidis, the droplet displaces itself along the tail to now occupy a position next to the annulus (*An*) of the tail. In the corpus epididymidis, the droplet is laterally displaced, while in the cauda epididy-

midis, many droplets are detached from the sperm. The saccular elements occupy one pole of the droplet but in living specimens they can be seen vortexing the circumference of the droplet in close apposition to the plasma membrane. The combined evidence for the morphological and biochemical presence of Golgi markers and lectin staining within the saccular elements of the droplet and modification of endogenous glycoproteins suggest that the saccular elements are modified Golgi/TGN components.

Figure 8. Low power light micrograph of adjacent seminiferous tubules of the testis immunostained with anti- β -COP (1:100 dilution). (a) The tubule in the lower half of the field represents stage XII of the cycle of the seminiferous epithelium. A prominent spherical dense immunoperoxidase reaction product (*large arrows*) is evident in the cytoplasmic lobe of the step 12 spermatids and corresponds to a staining of the spherical Golgi apparatus of these cells. Also evident is the well stained spherical Golgi apparatus (*arrowheads*) of the pachytene spermatocytes. The tubule in the upper half of the field represents stage VII of the cycle of the seminiferous epithelium. A dense immunoperoxidase reaction product in the form of spherical masses is present over the Golgi apparatus of pachytene spermatocytes (*arrowheads*). There is also intense staining of the hemispherical Golgi apparatus of step 7 spermatids (*small arrows*). Bordering the lumen (*Lu*), the cytoplasm of the late step 19 spermatids (*open arrows*) shows no staining other than that attributed to the methylene blue counterstain. (b) Higher power micrograph of stage VII. The late step 19 spermatids near the lumen (*open arrows*) show no staining with anti- β -COP unlike that seen over the Golgi apparatus of the step 7 spermatids (*arrows*) and pachytene spermatocytes (*arrowheads*). (c) Light micrograph of adjacent tubules of the caput epididymidis immunostained with anti- β -COP with a strong reaction over the Golgi apparatus (*G*) of the epithelial principal cells. Tails (*T*) of spermatozoa in the lumen (*Lu*) appear as discrete dense dots in cross section due to their inherent density. There is no staining of cytoplasmic droplets. Bars: (a) 40 μ m; (b and c) 20 μ m.

Figure 9. Light micrograph of tubules of the testis and caput epididymidis immunostained with ER marker antibody. (a) The tubule in the lower half of the field is at stage VII of the cycle, while that in the upper half is at stage VIII of the cycle. In either case, a uniform diffuse reaction is present throughout the entire epithelium with pachytene spermatocytes (*P*) being more intensely stained. At stage VIII, the cytoplasmic droplets of step 19 spermatids (*arrows*) are unreactive. (b) In the epididymis, the entire epithelium (*E*) is intensely reactive, while no reaction is present over spermatozoa or droplets in the lumen. Bars, 20 μ m.

evaluate the sugar linkages in the present study, these observations nevertheless indicate functional galactosyl and sialyl transferase enzymes as well as nucleotide sugar transporters in this cell-free system operating in the absence of detergent or cytosol. Furthermore, saccules of the droplet were stained by lectins (RCA I and HPL) specific for galactose and *N*-acetyl galactosamine, respectively. Having ruled out a saccular derivation from endocytosis the lectin staining serves to strengthen our argument that the saccules are Golgi elements.

A caveat to the identification of the saccules as a functional Golgi apparatus follows from the lack of immunoreactivity with β -COP, a constituent of Golgi coatamers implicated in the Golgi function, whose absence correlates in cell culture with merging of the Golgi apparatus with the ER (Lippincott-Schwartz, 1993). However, Hendricks et al. (1992) have shown that Brefeldin A in pancreatic exocrine cells leads to an alteration in Golgi structure, but with Golgi remnants clearly visible and not merged with the ER. Furthermore, although we were able to identify β -COP clearly in Golgi elements of spermatids and spermatocytes of the testis we were unable to find reaction associated with the well-developed and functionally active Golgi apparatus of Sertoli cells (Rambourg et al., 1979). Regardless, the absence of β -COP as well as the lack of detectable ER markers in the cytoplasmic droplet limits considerably interpretations as to the role for the saccules in protein transport.

All mammalian species conserve the droplet as a unique structure of the epididymal spermatozoa. The saccular elements were observed in living epididymal spermatozoa to encircle in a vortex-like fashion the circumference of the plasma membrane of the droplet. Indeed, Friend and Heuser (1981) have identified fusion intermediates of the saccular components of the cytoplasmic droplet with the plasma membrane by freeze-fracture EM. Our own observations supported this (Fig. 1 b) and, indeed, also revealed spot associations of individual saccules with each other.

During spermatozoa epididymal transit, it is firmly established that the sperm plasma membrane is modified by the addition or alteration of glycoproteins (Olson and Hamilton, 1978; Holt, 1980; Brown et al., 1983; Olson et al., 1987; see reviews by Eddy, 1988; Orgebin-Crist, 1987; Yanaginachi, 1988; Robaire and Hermo, 1988; Tulsiani et al., 1993), but the question remains how this is accomplished. Recent studies by Petruszak et al. (1991) and Nehme et al. (1993) demonstrated that in epididymal caput spermatozoa, the integral plasma membrane protein CE9 is restricted to the principal piece of the tail, while in cauda spermatozoa (i.e., after migration and loss of the droplet) (Fig. 10) CE9 is found throughout the plasma membrane. Many of these plasma membrane modifications occur during the distal migration of the cytoplasmic droplet along the sperm tail and before its loss in the cauda epididymidis (Fig. 10). Therefore, in view of the endogenous glycosylation capability of the saccular elements of the droplet, an intrinsic mechanism of sperm plasma membrane modification during epididymal transit is suggested. The isolation protocol for the saccules of the droplet as elaborated here should allow testing of this hypothesis in cell-free systems.

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