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Ordered Flow of Secretion From Accessory Glands to Specific Layers of the Spermatophore of Mealworm Beetles: Demonstration With a Monoclonal Antibody

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ABSTRACT Monoclonal antibodies were produced against the secretory product of the bean-shaped accessory gland (BAG) of male mealworm beetles (*Tenebrio molitor*). Antibodies from one clone (PL 6.3) recognized a 9,600 dalton protein with a pI of 6.6 which was found in homogenates of the BAG. The PL 6.3 antigen was first detected on Western blots of BAG proteins from 2-day adults, and amounts increased for the next 6 days until reproductive maturation was achieved. The antibody also recognized a polypeptide with a molecular weight (mw) of about 5,000 daltons which we believe to be derived from the larger 9,600 dalton antigen.

There are eight types of secretory cells in the BAG. By using light microscopic immunohistochemistry, we localized the antigens recognized by PL 6.3 in cell type 7 (intense staining) and cell type 5 (weak staining). Results from electron microscopic immunocytochemistry showed that antigen PL 6.3 was concentrated in the secretory granules characteristic of each of these two cell types and was absent in all other cell types.

PL 6.3 antigens were traced from the BAG into its secretory product and then into the prespermatophoric mass in the ejaculatory duct. The antigen was not randomly mixed with other secretory products of the accessory glands. As it flowed from the BAG and into the ejaculatory duct, it remained in a coherent, precisely localized mass. Within the definitive spermatophore, the PL 6.3 antigen was concentrated in discrete layers of material that line the lumen.

Spermatophores are packages for sperm transfer in many species of insects and other animals (Mann, '84; Tuzet, '77). Spermatophores are not homogenous in structure. Their various constituents are usually distributed into distinct zones, "bodies," and layers. How do insects assemble such complex multilayered structures? For arthropod cuticle, the order derives from the sustained secretion of chitin and proteins from epidermal cells and the rapid and repeated assembly of these products into laminae (Hepburn '85); thus new layers of cuticle are added one-by-one from the inside. Such a pulsed deposition of thin layers by apposition cannot account for the assembly of the spermatophore. The precursors of spermatophores are large secretory masses that collect in extracellular space and often flow some distance before they are transformed into the sperm sac.

Earlier morphological studies of the accessory glands of mealworm beetles (Dailey et al., '80; Dailey and Happ, '83) suggested a sequence of events that could lead to the assembly of the organized spermatophore. In this beetle, the eight cell types of the bean-shaped accessory gland (BAG) secrete their products in parallel to form a semisolid plug that flows into the ejaculatory duct where it is transformed into the spermatophore. Dailey et al. ('80) set forth the hypothesis, based on anatomy and on histological staining characteristics, that the secretions from different cell types are not blended together but remain segregated and self-coherent. Thus particular layers of the spermatophore might be derived from particular cell types in the accessory gland.

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To prove that blocks of secretion remain coherent from the cells of origin to the definitive spermatophore, one must have specific probes for one or more constituents of the secretion. Antibodies are obvious choices for the probes. There are at least 40 differentiation-specific proteins in the BAGs, and many of these proteins are also found in homogenates of the spermatophore (Happ et al., '82). However, most of the structural proteins are sparingly soluble and thus purification of a single immunogen is often difficult. The application of monoclonal antibody technology (which does not require rigorous antigen purification before immunization) can allow the production of specific antibodies to epitopes of the spermatophore proteins. In this paper, we describe one such monoclonal antibody which allows us to follow secretory epitopes from accessory gland cells to the walls of a spermatophore.

In order to minimize contamination of the heterogeneous immunogen with intracellular proteins, monoclonal antibodies were produced against antigens in the secretory plug dissected from the BAG. The present paper reports the localization, accumulation, secretion, and eventual fate of the antigens recognized by one monoclonal antibody (clone PL 6.3). Furthermore, it shows that the protein secretions, recognized by their epitopes, remain in coherent blocks that change in shape as the plug flows into the ejaculatory duct and finally is molded into the thin lining of the lumen of the spermatophore. Thus the immunochemical evidence supports the hypothesis that spermatophore assembly takes place by remolding of an ordered precursor mass as suggested earlier on purely morphological grounds.

MATERIALS AND METHODS

Mealworms were obtained from a commercial supplier and reared on Purina Chick Chow at 25°C. Newly emerged pupae were segregated, sexed, and kept at 26°C to insure uniform development. Adults were fed chick chow and potato and maintained at 25°C.

Accessory glands were dissected in phosphate-buffered saline (PBS) and homogenized in PBS or distilled water. Dried spermatophores were collected from the chick chow, homogenized in water, and spun at 15,000g in a microfuge. The supernatant contained water-soluble spermatophore proteins, the pellet containing water-insoluble proteins was washed twice. Protein levels were determined by the Lowry method (Lowry et al., '51) with bovine serum albumin (Sigma, St. Louis, MO) as the standard.

Slab gel electrophoresis (12% polyacrylamide) followed the methods of Laemmli ('70). Two-dimensional analysis involved an isoelectric-focusing tube gel followed by a sodium docecyl sulfate (SDS) slab gel, with sample preparation as described by Black et al. ('82). Prestained (Bethesda Research Laboratories, Gaithersburg, MD) and unstained (Sigma Chemical St. Louis, MO) molecular weight (mw) markers were used to calculate mw of unknowns by regression analysis. Western blotting was performed at 25 volts for 12 hr and immunodevelopment of the nitrocellulose sheets utilized 5% hybridoma supernatant in PBS as the primary antibody source (Towbin and Gordon, '84). The binding of the primary antibody was detected by adding rabbit antimouse IgG conjugated with horseradish peroxidase (Grimnes and Happ, '86). Solutions of primary and secondary antibody solutions contained 5% calf serum to reduce nonspecific binding.

Monoclonal antibodies were produced by slight modifications of the methods of Galfre and Milstein ('81), with SP2 cells as the parent myeloma. Details of the immunization, fusion, screening, and cloning procedures are presented in Grimnes and Happ ('86). Clones that were positive against insoluble proteins from spermatophores were detected by an enzyme-linked immunosorbant assay (ELISA). High titer ascites fluid was produced by injecting hybridomas into pristane-treated Balb/c mice.

During ELISA screening of hybridoma clones, clone PL 6.3 tested positively against spermatophore homogenate. Several other clones (PL7, PL9, PL13, and PL14) were recovered which produced antibody which was indistinguishable from that of clone PL 6.3 by our criteria of blotting patterns and immunohistochemistry. Antibody isolated from one of these alternate clones (PL7.2) was also used during the course of this work.

Immunohistochemical localization of antigen at the light microscopic level was achieved on paraffin sections of glutaraldehyde-fixed glands (0.5% in 0.1M phosphate buffer, pH 7.4). The primary antibody source was ascites fluid diluted 1/300 in PBS containing 2% rabbit serum (PBS-R). The second antibody was rabbit anti-mouse IgG-peroxidase conjugate (diluted 1/60 in PBS-R) with diaminobenzidine as the chromagen (Farr

and Nakane, '81).

For ultrastructural localization of PL 6.3 antigen, BAGs were fixed in phosphate buffer pH 7.2, containing either 4% formaldehyde and 0.1% glutaraldehyde or 1% formaldehyde and 0.5% glutaraldehyde; dehydrated through an alcohol series, and embedded in Epon. Thin sections (silver) were cut with a diamond knife on a Reichert Om-U2 microtome and collected on nickel grids. Sections were etched in 5% hydrogen peroxide for 5 min. After incubation in blocking sera, sections were incubated in primary antibody (purified from ascites fluid) for 30 min at room temperature, in biotinylated anti-mouse IgG for 30 min, in avidin-biotin peroxidase conjugate for 60 min (Vectastain, Vector Labs, Burlingame, CA), and they were stained with diaminobenzidine for 10 min (Hsu et al., '81; Childs, '83). Controls for the immunohistochemical study included treatment with nonimmune sera and omission of primary or secondary antibody. Sections were examined on a Philips 201 electron microscope at 40 kV.

RESULTS

PL 6.3 antigen identity and organ specificity

Homogenates of the secretory plug from the BAG were injected into mice and hybridomas were produced by fusion of mouse lymphocytes with myeloma cells as discussed in Materials and Methods. During ELISA screening of hybridoma clones, the clone designated PL 6.3 tested positively against spermatophore homogenate.

When PL 6.3 supernatant was applied to a blot from an SDS gel containing homogenates of the BAG, the tubular accessory gland (TAG), and the spermatophore, the antibody recognized a major band in the BAG and in the spermatophore (Fig. 1A). The antigen's mw was 9.6 kd (SE 0.5 kd) as determined by regression analysis. The greatest part of the spermatophore antigen was present in the water-insoluble portion of the spermatophore (well 4) rather than the water-soluble fraction (well 5). Additional homogenates of secretory plug, seminal vesicles, testis, fat body, and hemolymph were tested for antigen content (Fig. 1A). No antigen could be detected in seminal vesicle (well 6), testis (well 7), hemolymph (well 8), or fat body (well 9). The PL 6.3 antigen appears to be produced in the BAG (well 2), secreted into the secretory plug within the BAG lumen (well 3), and molded into part of the spermatophore (well 4).

A second, less intense antigen band of lower molecular weight (about 5 kd) was often detected in BAG, plug, and spermatophore samples. We suspected that the presence of this second antibody-reactive band might reflect genetic polymorphism within the population, as has already been described for certain secretory proteins of the TAG (Grimnes and Happ, '85) and the BAG (Grimnes and Happ, '86). Alternatively, the two bands might be derived from different types of secretory cells in the epithelium of the glands. To determine if the observed differences in mobility were the result of genetic variation, blots of individual glands were probed with antibody PL 6.3. To test for regional differences in antigen production, homogenates of anterior and posterior halves of several BAGs were compared. Each BAG sample contained the slower migrating band and a small amount of faster migrating (lower mw) band. No variation in the mw or relative amounts of either antigen was seen among individuals or between anterior and posterior portions of a single gland (data not shown).

The lower antigen band was sometimes absent in blots of the BAG homogenates, but it was consistently detected in samples that are difficult to collect (such as secretory plugs from the lumen of the glands) or in those samples left at room temperature for many hours (e.g., dried spermatophores). We thought that the band might represent a breakdown product of the 9.6 kd protein. To test this hypothesis, secretory plugs were dissected in the presence and absence of 10^{-4} M phenyl methyl sulfonyl fluoride (PMSF), a potent protease inhibitor. Relative amounts of the two bands present in each sample were estimated by running a photographic negative of the blot through a densitometer and measuring the area under the curve for each protein. With PMSF, only 14% of the antigen was present as the lower mw band. Without PMSF, the lower band was 36% of the total antigen amount. Therefore, the lower mw antigen band appears to be a breakdown product of the 9.6 kd protein which retains its active epitope.

On Western blots of BAG homogenates from pupae and adults, the PL 6.3 antigen was present in adult BAGs but not in pupal BAGs, and thus it is differentiation-specific (Fig. 1B). Both 5 kd and 9.6 kd antigen bands appeared as early as the 2-day post-ecdysial adult stage. Staining intensity increased

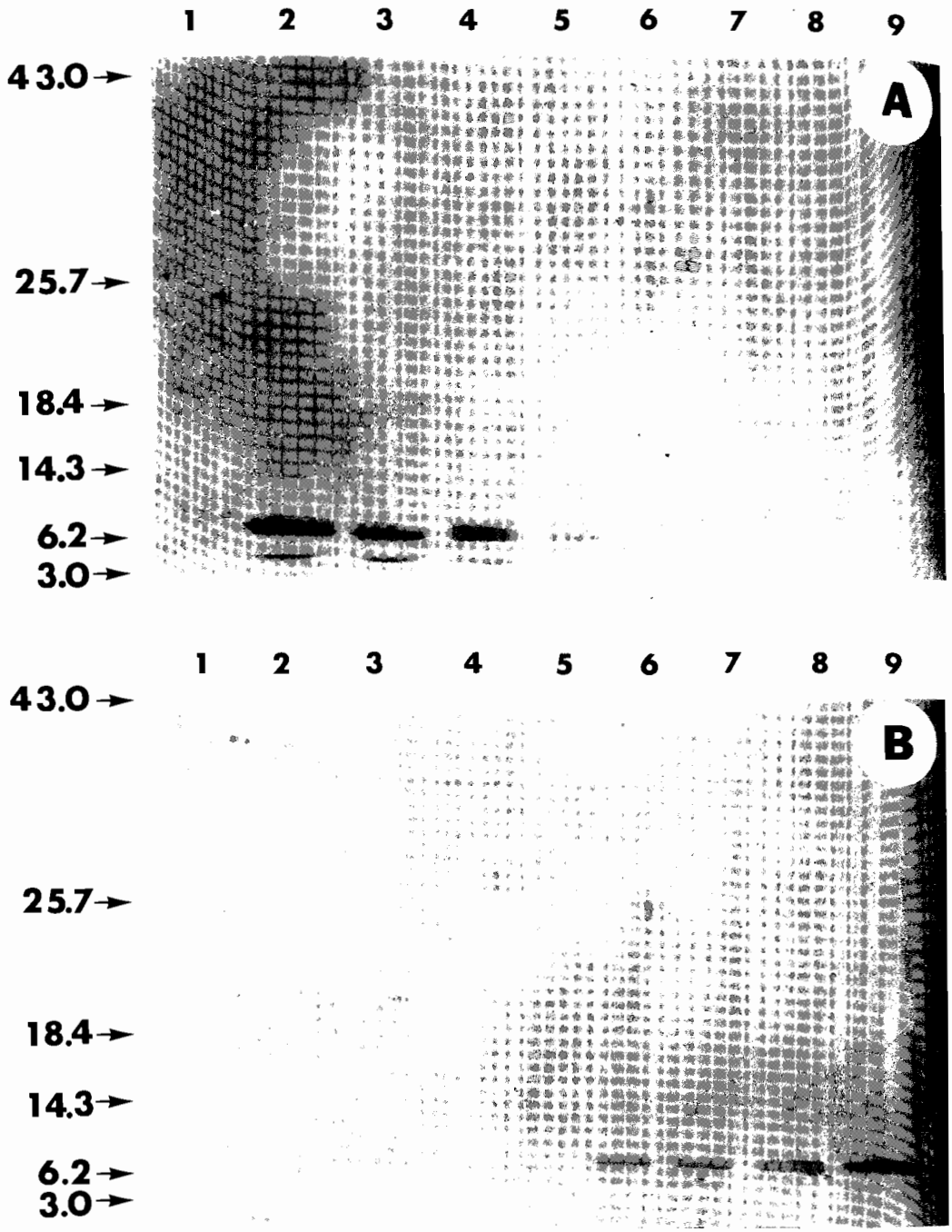

Fig. 1. A) Western blot of SDS polyacrylamide gel to show the organ specificity of PL 6.3 antigens. The sample homogenates were applied to wells at the top of the gel. 1) TAG; 2) BAG; 3) secretory plug; 4) water-insoluble spermatophore; 5) water-soluble spermatophore; 6) seminal vesicle; 7) testis; 8) hemolymph; and 9) fat body. Each well contained 100 μg total protein. Molecular weights are in kilodaltons. B) Western blot of an SDS-polyacrylamide gel of homogenates of BAGs of increasing ages to show the appearance of the PL 6.3 antigens with terminal differentiation. BAGs were dissected from: 1) 0-day pupae; 2) 4-day pupae; 3) 6-day pupae; 4) 8-day pupae; 5) 0-day adult; 6) 2-day adult; 7) 4-day adult; 8) 6-day adult; and 9) 8-day adult. Each well contained 100 μg total protein. Molecular weights are in kilodaltons.

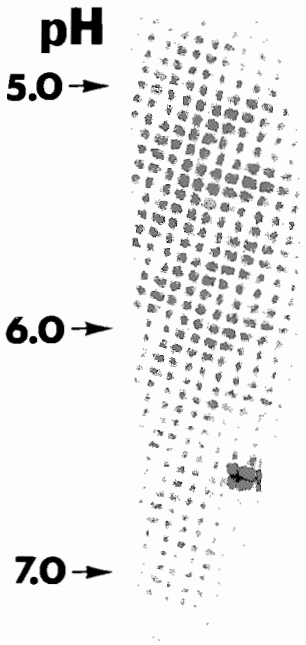

Fig. 2. Western blot of an isoelectric focusing tube gel to which BAG homogenate had been applied. Immunodeveloped with PL 6.3 antibody.

through the 6-day adult stage and remained at a constant level in older animals. Synthesis probably begins earlier than the 2-day adult stage, since trace amounts of antigen can sometimes be seen as early as the 8-day pupal stage in immunohistochemical studies (see below).

When isoelectric focusing tube gels were electroblotted, only one band at a pI of 6.6 was recognized by PL 6.3 antibody (Fig. 2). On two-dimensional gels (IEF tube, SDS slab), PL 6.3 antigen had a similar isoelectric point 6 and was located near the bromphenol blue front.

Immunohistochemical localization of PL 6.3 antigen in the BAGs

Treatment of paraffin sections of the BAGs with PL 6.3 antibody followed by secondary antibody-peroxidase showed that PL 6.3 antigens were confined to specific regions within the secretory epithelium. Staining with diaminobenzidine was very strong around the inner curve of each of the paired BAGs, a location which corresponds to cell type 7 (Fig. 3B,C). In addition, weak but consistent staining was detected in posterior regions containing cell type 5 (Fig. 3C). The staining of cell type 5 was only slightly more intense than nonspecific staining in surrounding areas but it was absent from control slides (Fig. 3A).

After standard fixation with 3% glutaraldehyde followed by osmium tetroxide, the secretory granules of each cell type are distinguishable by their characteristic morphology. The granules of cell type 5 are granular while those of cell type 7 contain membranous whorls (Dailey et al., 1980). With the gentler fixation schedule used for immunocytochemistry, morphological preservation was not optimal (e.g., Fig. 6C), yet antigens in both cell types 5 and 7 of the BAG were precisely localized within the secretory granules of the cells (Fig. 4). Staining was uniform across the entire granule of each cell type, showing no apparent compartmentalization of antigen within the granule and very little antigen was detected in the surrounding cytoplasm of these cells (Fig. 4C,F). In some cases, the membranous whorls of the granule within type 7 cells were disrupted and apparently extracted. Immunological staining of type 7 granules occurred only where membranes persisted; therefore, antigen PL 6.3 appears to be associated with the membranous whorls. Preservation of cell type 5 was more uniform; extraction of this granule type was not observed.

Immunochemical localization of PL 6.3 antigens in extracellular space

At the light microscopic level, intensely stained materials were found in discrete patches of the secretory plug within the lumen of the BAG. The staining in the secretory plug was along its medial edge—a location associated with secretions of type 7 cells (Dailey et al., '80). In addition, well-defined staining was seen in the prespermatophoric mass which forms as the plugs from the bilateral glands fuse medially within the ejaculatory duct. The shape and position of the antigen-positive secretion changed as it flowed backwards. At the point of fusion of the plugs from the BAGs, the positive reaction was confined to a thick zone in the ventral part of the ejaculatory duct (Fig. 3D). As the material passed posteriorly, the patch of secretion appeared thinner and spread laterally and dorsally (Fig. 3E), forming a U-shaped layer of the prespermatophoric mass in Figure 3F.

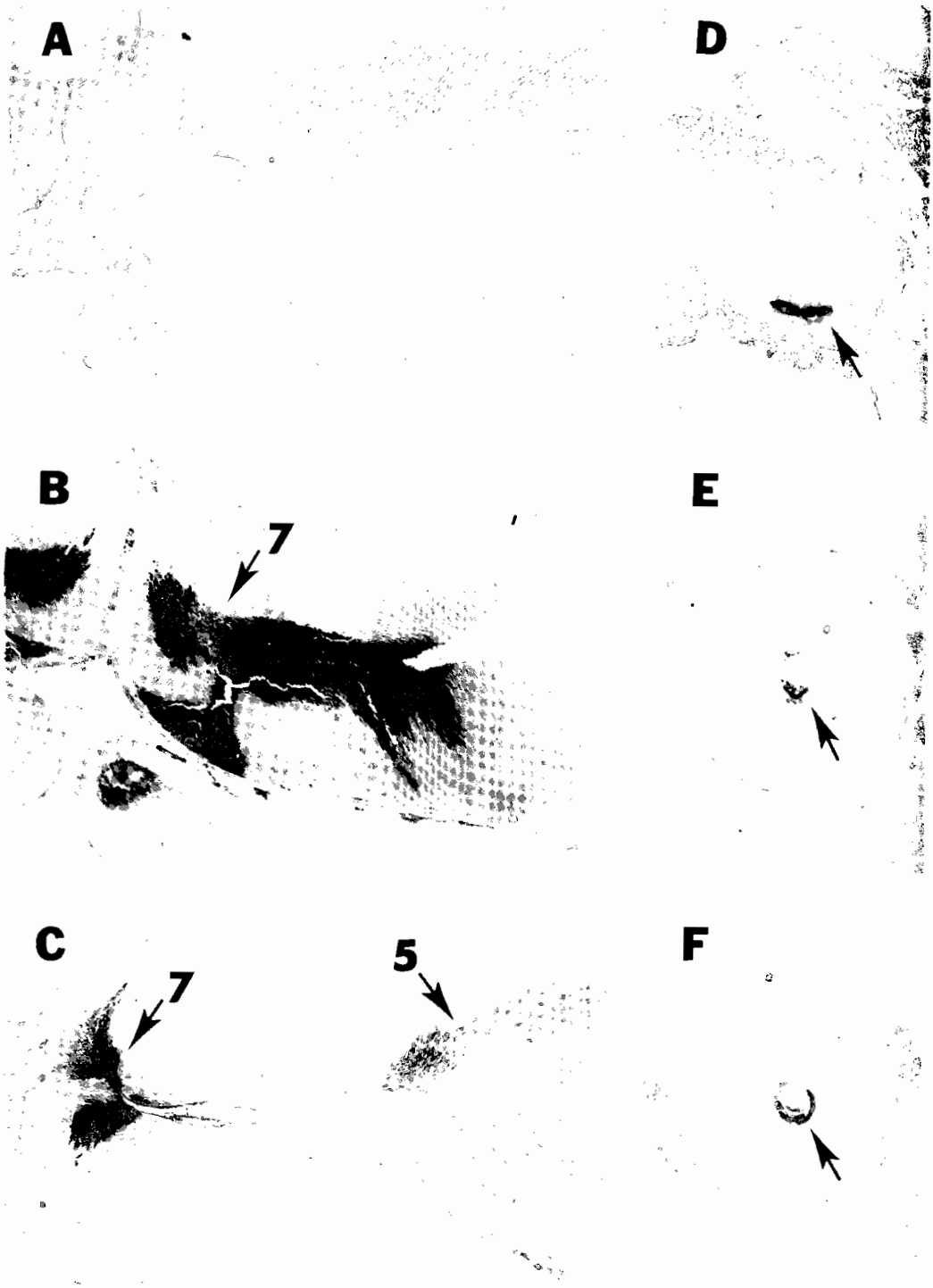

Fig. 3. Light micrographs showing immunohistochemical demonstration of the PL 6.3 antigens in the BAG and its secretory products. A) Control section of the BAG, no primary antibody (× 300). B–C) Progressively posterior sections from the gland, cell types of the immunological reactions are as marked (× 300). D–F) Transverse sections through the ejaculatory duct, showing localization of PL 6.3 antigen in the prespermatophoric mass (× 300). D shows the fusion product of the two secretory plugs from the right and left BAGs at the anterior end of the ejaculatory duct. E and F are sections approximately 0.7 mm and 0.85 mm posterior to D, respectively.

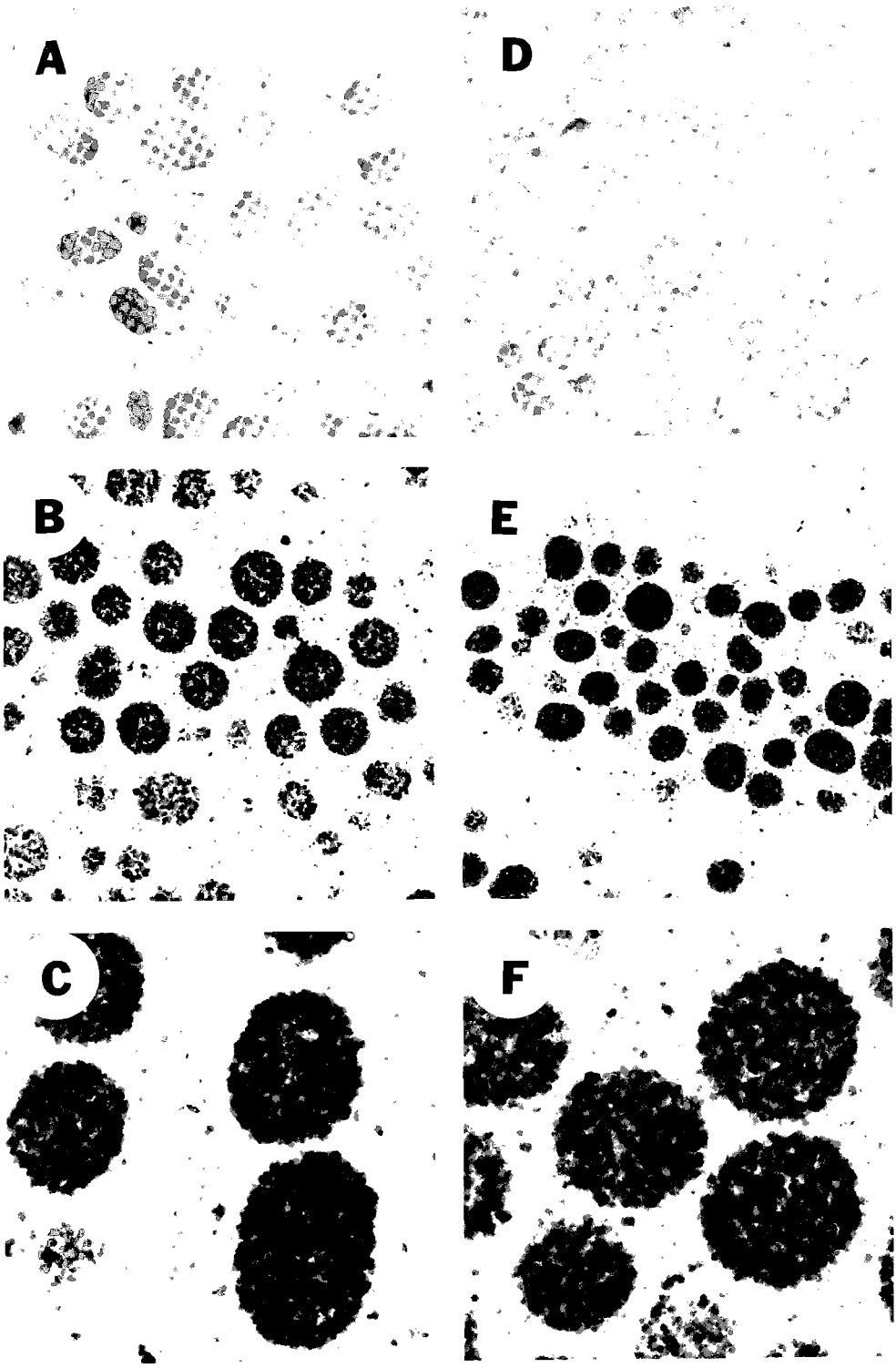

Fig. 4. Electron micrographs showing the immunocytochemical localization of PL 6.3 antigen in thin sections of different cell types of the BAG: A) type 5, no primary antibody (× 14,000); B) type 5, PL 6.3 antibody (× 14,000); C) type 5, PL 6.3 antibody (× 33,500); D) type 7, no primary antibody (× 14,000); E) type 7, PL 6.3 antibody, (× 14,000); and F) type 7, PL 6.3 antibody (× 33,500).

The antigen recognized by PL 6.3 was restricted to specific layers in the spermatophore ejected from the ejaculatory duct. At the light level, strong immunochemical staining was seen at the inner and outer margins of the lumen (Fig. 5B). Additional faint immunochemical staining was sometimes seen in the central core of the spermatophore. In a previous report (Bricker and Happ, '85), we showed that the lining of the spermatophore lumen (Fig. 6A,B) resembled the whorled membranes of type 7 granules (Fig. 6C). With immunocytochemical procedures, PL 6.3 antigens, characteristic of cell type 7, are likewise confined to the lining of the lumen (Fig. 5C–E). At both the light and electron microscopic levels, all immunocytochemical evidence is consistent with flow and molding of a block of secretion into a thin hollow cylinder within the ejaculatory duct that subsequently splits to coat the lumen of the spermatophore.

DISCUSSION

Nature of the PL 6.3 antigen

In many accessory reproductive glands, most of the differentiation-specific proteins seem to be synthesized within the glands themselves (Chen, '84; Happ, '84). In other species, some proteins which have been synthesized in organs like the fat body are transported through the hemolymph and are taken up by the accessory glands (Friedel and Gillott, '76; Peferoen and De Loof, '84). For the BAGs of *Tenebrio molitor*, PL 6.3 antigen is apparently produced only within the accessory gland. There is no evidence that this protein is present in any other tissue.

One major protein (mw 9.6 kd) and one minor protein (mw 5 kd) were detected on Western blots probed with PL 6.3 antibody. Are these bands related to one another, and if so, how? There are four possible explanations: 1) the two bands could be the products of two allelic genes, which are distributed among the animals in the population; 2) one or both antigens could be cell-specific—produced by only cell type 5 and/or cell type 7; 3) the 9.6 kd antigen could be the biologically important molecule and the 5 kd antigen could be an artifactual consequence of degradation during sample preparation; or 4) the 5 kd antigen could be the biologically important molecule which is derived by processing a 9.6 kd precursor.

We found no variation when we compared the bands recognized by PL 6.3 among different animals. To test for cell-specificity of the two antigens, we compared homogenates from the anterior regions of the BAG with those from the posterior regions of the glands. Cell type 7 occurs in both anterior and posterior regions of the BAG, while cell type 5 is confined to the posterior regions (Dailey et al., '80). Both bands are found in both regions on Western blots of the tissue homogenates. Thus type 7 cells apparently give rise to both bands. We cannot exclude the possibiility that the type 5 cells might give rise to one band or both or neither. Type 5 is a minor cell type (Dailey et al., '80) and it is possible that we failed to detect the epitope-bearing products of type 5 cells on the Western blots simply because of their relative scarcity. In our immunohistochemical work reported above, we found strong staining of type 7 products in the secretory plug but we failed to detect weakly staining antigens at the zone characteristic of type 5 products (Dailey et al., '80). We know that immunocytochemistry is more sensitive than Western blotting for the detection of the antigen in late pupal glands (unpublished results). We suspect that type 7 products account for almost all of the intense extracellular staining.

Our data are consistent with a precursor-product relationship between the 9.6 kd antigen and the 5 kd form. In fresh spermatophores, the 9.6 kd band is always the prominent one. The smaller antigen is increasingly pronounced in samples that have aged physiologically, i.e., homogenates of extracellular products and those of cast spermatophores. Furthermore, the lower band becomes more prominent with prolonged storage of homogenates, and this apparent degradation is reduced by inclusion of PMSF in the dissection buffer. If the shift from the 9.6 kd form to the smaller species were part of a physiological maturation of the secretion, then we would expect the younger animals, which are not yet reproductively mature, to be relatively richer in the 9.6 kd form and poorer in the 5 kd form than the older animals, but we could find no such shift as the animals matured (Fig. 2). No evidence argues for a required cleavage from a 9.6 kd precursor to produce a physiologically active 5 kd product. The simplest explanation is that instability of the 9.6 kd secretory antigen gives rise to the 5 kd band. More specific information on the relationship between these antigens and on the breakdown of the 9.6 kd antigen can be obtained from epitope

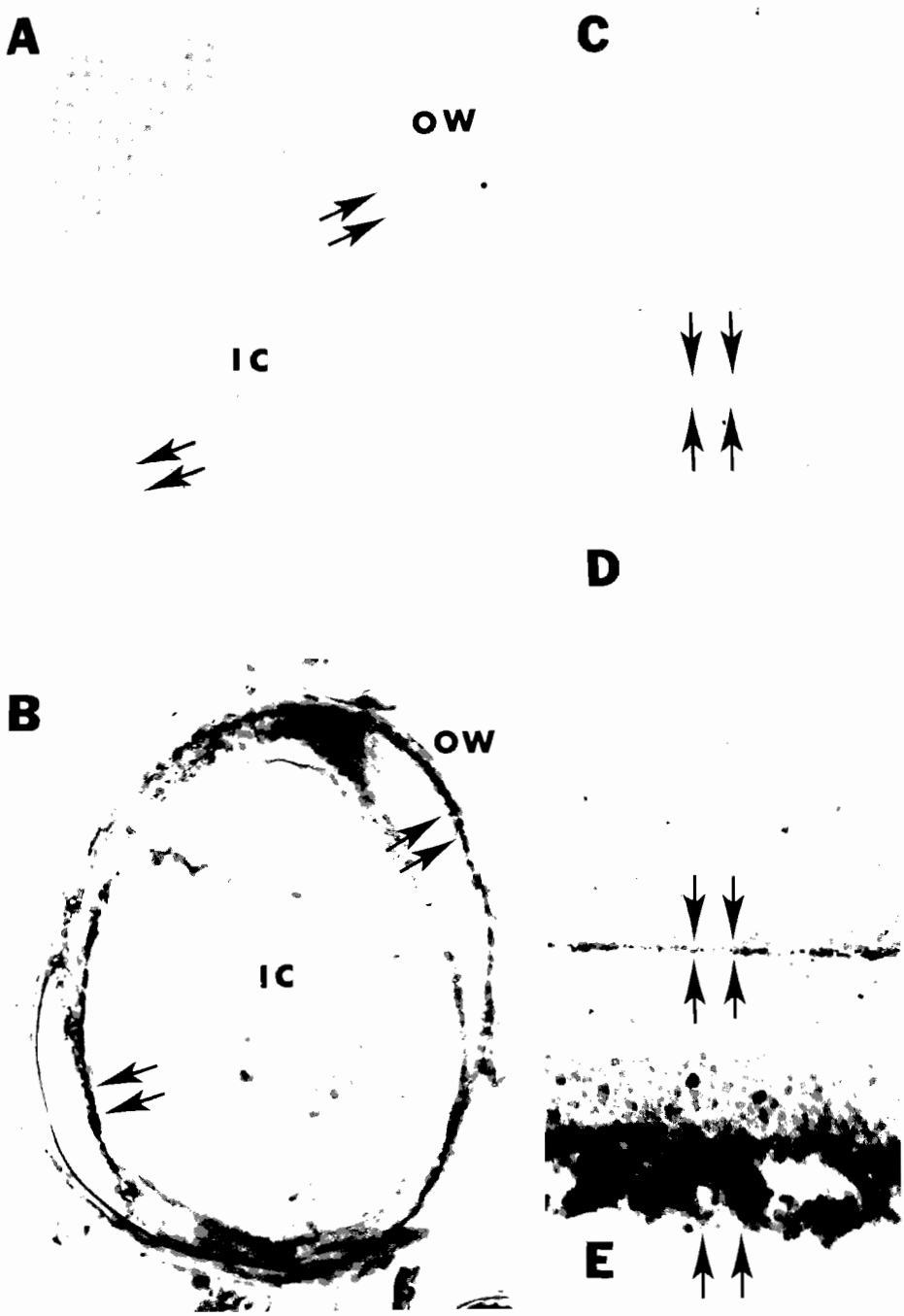

Fig. 5. Immunohistochemical localization of PL 6.3 antigen in the spermatophore at the light microscopic level (A–B) and the electron microscopic level (C–E). A) No primary antibody (× 1,300); B) PL 6.3 antibody, staining reaction on inner surface of OW and outside of IC (× 1,300); C) control, no primary antibody (× 14,000); D) PL 6.3 antibody (× 14,000); E) PL 6.3 antibody (× 50,000). Immunoreactive layer is between the arrows.

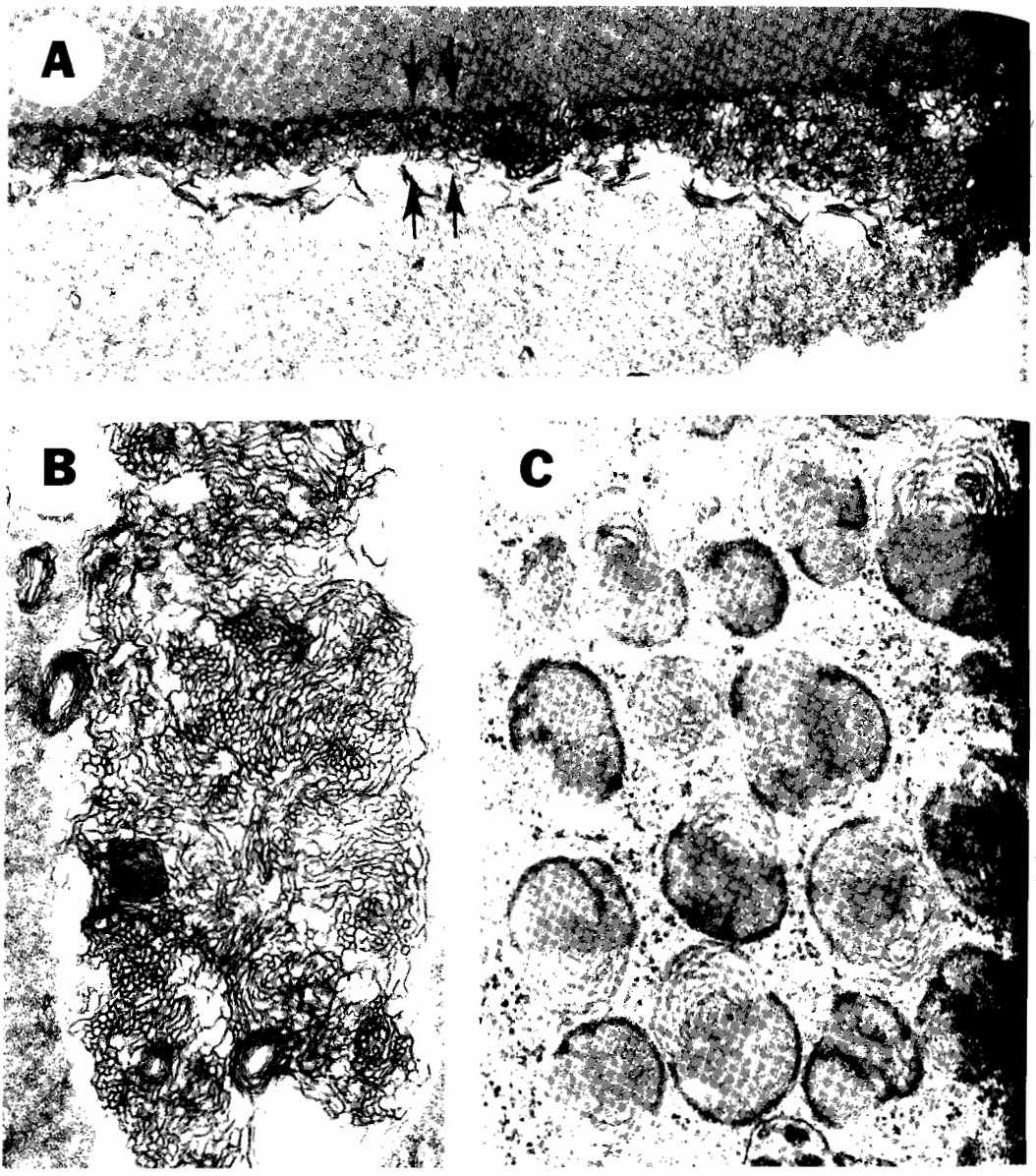

Fig. 6. A) Transmission electron micrograph of wall of the spermatophore. The membranous lining of the lumen (between the arrows) corresponds to the immunoreactive layer shown in Figure 5D. Finely granular secretion fills the lumen. Fixation in 3% glutaraldehyde, 1% osmium tetroxide. (× 13,000). B) Transmission electron micrograph of the lining of the lumen of the spermatophore. Fixation in 3% glutaraldehyde, 1% osmium tetroxide. (× 20,000). C) Transmission electron micrograph of the type 7 granules in the BAG. Fixation in 1% paraformaldehyde, 0.5% glutaraldehyde (× 35,000).

mapping, where experimentally produced fragments are probed with this monoclonal antibody.

Antigen PL 6.3 was identified in two-dimensional gels, migrating to a pI of 6.6. This pI is more basic pH than that of prominent proteins of about 10 kd that were identified as differentiation-specific (spots 10, 11, and 12 in Happ et al., '82). However, a minor tritiated protein spot, perhaps corresponding

to PL 6.3 antigen, first incorporates detectable amounts of leucine in 4-day post-ecdysial adults (Fig. 8 in Happ et al., '82). In the present report, we detected antigen PL 6.3 in 2-day adults with Western blots and in the 8-day pupae using immunohistochemical assays. Thus, the PL 6.3 antibody is a suitable probe for early and specific detection of the PL 6.3 protein which appears to be a marker for the onset of terminal differentiation.

Ordered flow of secretory materials

Many spermatophores appear to be formed from patchy secretory masses (Happ, 84). We believe that the final organization of the spermatophore is linked to the patchiness in the secretory masses. A given cell type might secrete different products in a temporally programmed series, or a variety of different cell types each might secrete their individual products in parallel. Parallel secretion occurs in *Tenebrio*. The ordered secretory mass subsequently undergoes rearrangement during its transformation into the spermatophore. Monoclonal antibodies like PL 6.3 provide powerful tools to follow specific secretory proteins through the process of spermatophore formation. To our knowledge, there have been no other reports that unequivocally demonstrate linear flow and nonrandom mixing of cell-specific products after secretion and during spermatophore formation.

In this study, we have described an antibody which recognizes an epitope present in granules of cell types 5 and 7. Cell type 7 granules are most intensely stained and form a coherent mass of secretory material which runs along one edge of the secretory plug in the BAG. Downstream, the PL 6.3-positive mass remains discrete. As the secretions flow down the ejaculatory duct, spiral arrays of cuticular spines found in the wall of that duct might retard backward flow and somehow drag blocks of secretion around the outer edge of the prespermatophoric mass (Fig. 3). In the completed spermatophore, PL 6.3 antigen is in the thin layer that lines the lumen.

As we learn more about molding of the prespermatophoric mass in the ejaculatory duct and its subsequent transformation in the female's reproductive tract, we hope to determine the precise physiological role of each constituent in the secretions of the accessory glands. From the present study, we know that antigens from cell type 7 form a continous layer around the lumen of the spermatophore. What might be the special roles of these antigens from cell type 7? Two possibilities suggest themselves: a role in spermatophore formation and a role in maintaining a special environment for sperm maintenance. In the first instance, it may be that the membranous whorls from an easily ruptured zone that splits to admit seminal fluids and sperm as they are forced into the prespermatophoric mass. In the second instance, the osmiophilic (lipoid?) secretions of cell type 7 may form a permeability barrier that seals the sperm away from osmotic and ionic changes in the environment of the spermatophore. Finally, it is possible that the PL 6.3 antigen functions in sperm activation, maturation, and nutrition but this role seems unlikely in view of its insoluble nature. At present, we can only speculate about the physiological importance of the PL 6.3 antigens. Nevertheless, the PL 6.3 antibody has allowed us to show that self-coherent secretions from one cell type are molded into one layer of the final product for the spermatophore of *Tenebrio*. This strategy for assembly of an ordered structure in extracellular space may be commonly used by arthropods.

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