The Pupal Cuticle of *Drosophila*: Differential Ultrastructural Immunolocalization of Cuticle Proteins

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Abstract. Precise ultrastructural localization of *Drosophila melanogaster* pupal cuticle proteins (PCPs) was achieved by the immunogold labeling of frozen thin sections. PCPs were found in lamellate cuticle and intracellular vesicles but, curiously, were absent from the assembly zone of the cuticle. Antibodies that distinguish between the two classes of PCPs—low molecular weight (L-PCPs) and high molecular weight (H-PCPs)—revealed that the morphologically distinct outer lamellae contained L-PCPs and the inner lamellae contained H-PCPs. The sharp boundary between these two antigenic domains coincides with the transition from the outer to the inner lamellae, which in turn is correlated with the cessation of L-PCP synthesis and the initiation of H-PCP synthesis in response to 20-hydroxyecdysone (Doctor, J., D. Fristrom, and J. W. Fristrom, 1985, *J. Cell Biol.* 101:189–200). Hence, differences in protein composition are associated with differences in lamellar morphology.

THE insect cuticle is an extracellular exoskeleton secreted by the underlying epidermis and consists of an outer epicuticle and a lamellate region composed mainly of chitin and protein (Neville, 1975). The repeated shedding (ecdysis) and secretion of cuticles provide the insect with the opportunity to change form during development. *Drosophila melanogaster* has three larval stages, one pupal, and one adult stage, each of which has a morphologically and/or chemically distinct cuticle. Furthermore, except for the first two larval stages, the protein composition differs extensively among stages, presumably reflecting the varied functions of the different cuticles (Chihara et al., 1982). The lamellate region of the pupal cuticle is secreted from about 9 to 18 h after puparium formation. Transmission electron micrographs reveal that, as in many insects, the lamellate cuticle is composed of two zones, an outer zone consisting of dense irregular lamellae deposited before pupation, and an inner zone consisting of less dense, more regular lamellae deposited after pupation (Doctor et al., 1985).

Pupal cuticle formation is particularly amenable to study because it is formed by imaginal discs cultured in vitro under a proper regimen of the steroid hormone, 20-hydroxyecdysone (Fristrom et al., 1982). A combination of in vivo and in vitro studies has demonstrated that the imaginal epidermis in *Drosophila melanogaster* exhibits a biphasic pattern of pupal cuticle protein (PCP)[1] synthesis and deposition (Doctor et al., 1985). About 9 h after puparium formation low molecular weight (8,000–25,000) PCPs (L-PCPs) are synthesized and secreted. At 12 h a small rise in 20-hydroxyecdysone titer apparently causes synthesis of the L-PCPs to stop, and synthesis and deposition of high molecular weight (40,000–82,000) PCPs (H-PCPs) to begin. This switch in PCP synthesis coincides with a change in cuticular lamellar structure such that L-PCP synthesis occurs during deposition of the outer lamellae and H-PCP synthesis occurs during deposition of the inner lamellae. These results suggest that the inner and outer lamellae have different protein compositions (Doctor et al., 1985). However, it is possible that deposition of PCPs may not be directly dependent on their synthesis. For instance, L-PCPs synthesized early could be stored and secreted later and H-PCPs could be deposited throughout the entire thickness of the cuticle by intussusception. The main purpose of the experiments reported here was to determine the ultrastructural location of L-PCPs and H-PCPs within the cuticle using antibodies previously shown to distinguish between the two classes of PCPs (Silvert et al., 1984 and Doctor et al., 1985). We used immunogold labeling of frozen thin sections because, as compared with other techniques, it allowed more precise antigenic localization while providing adequate preservation of ultrastructure. By this means we have demonstrated that the sequence of synthesis of PCPs parallels the sequence of deposition and that the two morphological domains of the cuticle have distinct protein compositions.

Materials and Methods

Fly Cultures

White puparia were collected from cultures of mass-reared Oregon R larvae (Mitchell and Mitchell, 1964) and were maintained at 25°C for varying periods.

[1] *Abbreviations used in this paper:* PCP, pupal cuticle protein; H-PCP and L-PCP, high and low molecular weight PCP, respectively.

Antibodies

The various antisera were made against urea soluble proteins extracted from cuticles. The complex rabbit anti-PCP antiserum, which was absorbed against unevaginated discs without cuticle, recognizes virtually all the soluble PCPs (Fig. 1, lane *2*) apparent on one-dimensional gels (Doctor et al., 1985). The complex rabbit anti-larval cuticle protein antiserum (Silvert et al., 1984) cross-reacts strongly with most L-PCPs but only weakly with H-PCPs (Fig. 1, lane *3*). The mouse monoclonal antiserum (MAB 82, an IgG) is monospecific for PCP 82 (Fig. 1, lane *4*) (Doctor et al., 1985).

Electron Microscopy

The method for preparing Epon-embedded tissue has been previously described (Doctor et al., 1985). For cryoultramicrotomy (frozen thin sections) pupae were removed from the pupal cases, and the heads and thoraces were fixed in 1.75% glutaraldehyde, 0.5% formaldehyde (Ladd Research Industries Inc., Burlington, VT) in 0.1 M cacodylate buffer (pH 7.6) at room temperature for 2 h. The fixed tissue was infiltrated with 80% sucrose as a cryoprotectant in 0.1 M cacodylate buffer for either 1 h at room temperature or overnight at 4°C. Small pieces of integument were frozen onto copper chucks by rapid immersion in liquid nitrogen. Blocks were stored in liquid nitrogen until sectioned.

The methods of sectioning and staining were similar to those of Tokuyasu (1984). Sections were cut on a Sorvall MT-2B ultramicrotome with an LTC-2 (Sorvall Instruments Div., E. I. DuPont de Nemours & Co. Inc., Newtown, CT) freezing attachment at −90°C on a dry glass knife. Sections were transferred on a drop of 80% sucrose onto parlodian-coated grids.

Sections were blocked for at least 0.5 h with 1% bovine serum albumin (BSA) in 0.02 M Tris (pH 7.6), and 0.1% sodium azide and were then washed by transfer through three drops of wash solution (0.1% BSA, 0.02 M Tris, pH 7.6, 0.1% sodium azide) before incubation in the primary antibody solution for 1 h at room temperature. The rabbit antisera were diluted 1:3,000; the monoclonal culture supernatant was used undiluted. In double-label experiments 3 μl rabbit antiserum diluted 1:10 was added to 997 μl MAB 82 culture medium. Washed grids were incubated for 1 h in either goat anti-rabbit IgG conjugated to 5-nm gold particles diluted 1:10 in wash buffer or goat anti-mouse IgG conjugated to 20-nm gold diluted 1:5 (Janssen Pharmaceutica, Beerse, Belgium). After treatment with antibodies grids were washed and stained in neutral uranyl acetate, embedded in 0.8% methylcellulose (Tokuyasu, 1984), air dried, and viewed with a JEOL electron microscope at 60 kV.

Results

Localization of PCPs

The three major layers of the pupal cuticle—epicuticle, outer lamellae, inner lamellae—are sequentially secreted by the epidermis from 3–18 h after pupariation (Fig. 2). After formation of the epicuticle (3–8 h), dense outer lamellae are deposited until 12 h. Next, less dense and more regular inner lamellae are formed between 12 and 18 h. Always intervening between the most basal lamellae and the epidermis is the assembly zone (Delbecque et al., 1978) (Fig. 2), the presumed site of self-assembly of cuticular precursors into lamellae (Neville, 1975).

PCPs were localized in the thoracic integument of 15.5-h-old pupae with a complex rabbit antisera known to react with all the major PCPs (Fig. 1, lane *2*). Fig. 3*a* shows that the antiserum binds to both inner and outer lamellae. In addition, binding occurs to cytoplasmic vesicles (Fig. 3*b*), which suggests that cuticle proteins are being transported to the apical surface of the cell for exocytosis. Labeling was absent or reduced in the assembly zone.

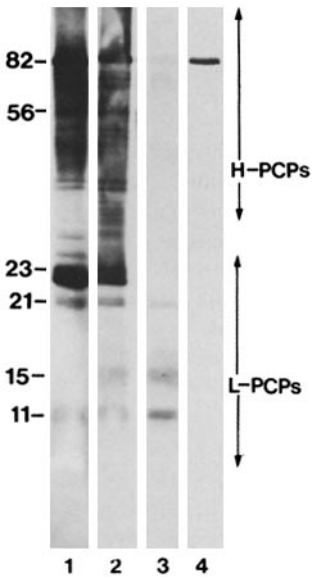


Figure 1. Protein gel blot of SDS-soluble PCPs (5 μg in each lane) probed with the various antisera used in the study. Antibodies were detected by peroxidase as described by Doctor et al. (1985). Lane *1*, India ink–stained proteins. Lane *2*, anti-PCP antiserum. Lane *3*, anti-larval cuticle protein antiserum. Lane *4*, monoclonal antibody to PCP 82. Molecular weight (thousands) of selected proteins is indicated on the left, and the range of L-PCPs versus H-PCPs is indicated on the right.

Differential Labeling of Inner and Outer Lamellae

PCP 82, a representative H-PCP, was localized using MAB 82 (Fig. 1, lane *4*). In 18-h pupae only the inner lamellae showed a positive reaction (Fig. 4*a*). In 11-h-old pupae, before synthesis of PCP 82, no labeling of the cuticle was observed (data not shown).

To localize L-PCPs, a complex rabbit antiserum, originally made against larval cuticular proteins, was used (Silvert et al., 1984). This antiserum reacts strongly with the outer lamellae in 18-h pupae (Fig. 4*b*). Low levels of labeling over the inner lamellae probably do not reflect specific binding of antiserum to L-PCPs because (*a*) similar background levels of randomly distributed gold particles were seen over the cytoplasm in contrast with the specific binding of anti-PCP antiserum to epidermal vesicles (Fig. 3*b*); and (*b*) the Western data (Fig. 1, lane *3*) showed a weak cross-reactivity to some H-PCPs that are present only in the inner lamellae (Fig. 4*a*).

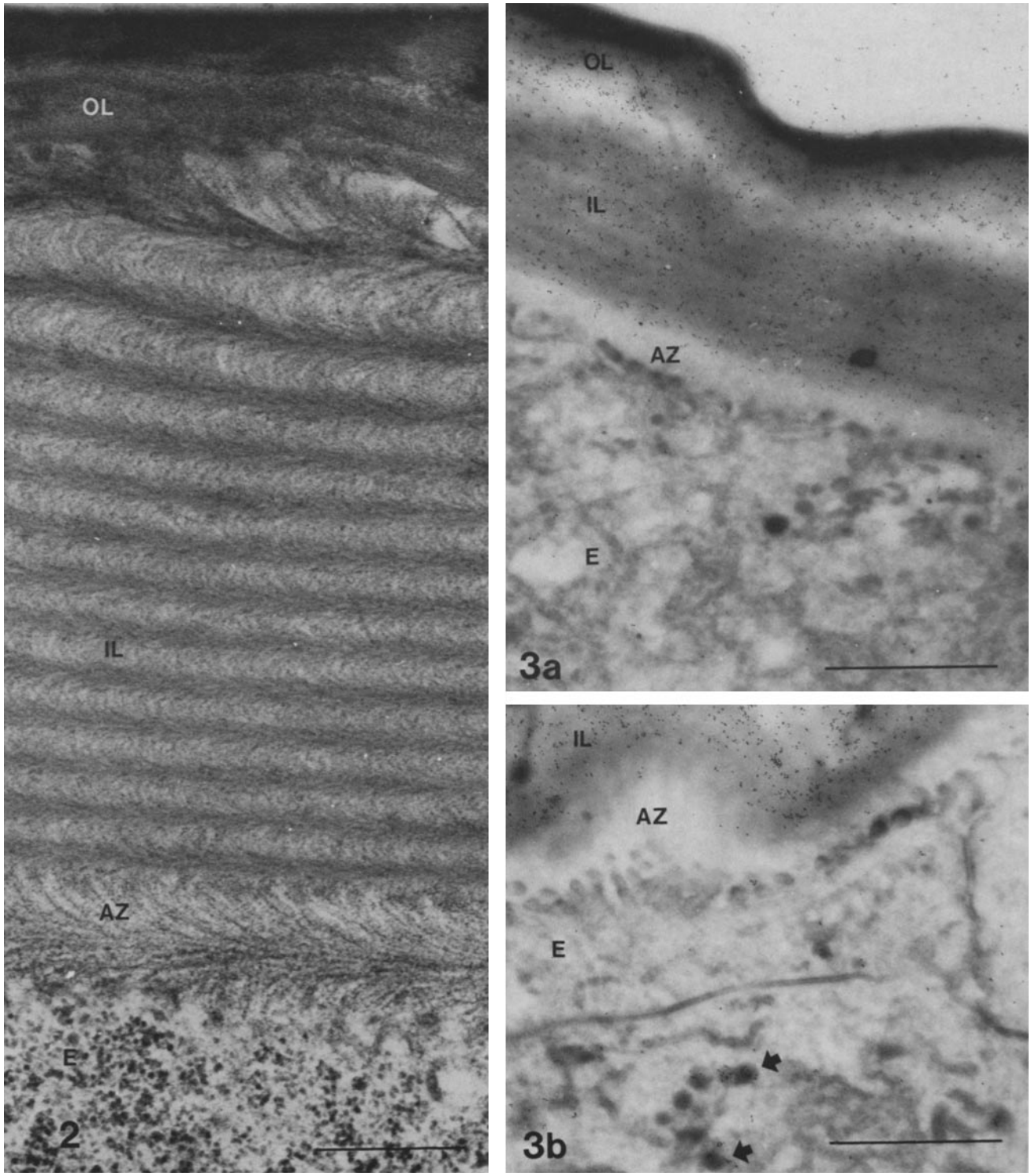
To confirm that PCP 82 and L-PCPs occupy mutually exclusive domains within the cuticle, sections were simultaneously labeled with MAB 82 to localize PCP 82 and with anti–larval cuticular protein antibody to localize L-PCPs (Fig. 5). The antibody binding to PCP 82 was visualized with goat anti–mouse antibody conjugated to 20-nm gold particles, and antibody bound to L-PCPs was visualized with goat anti–rabbit antibody conjugated to 5-nm gold. A sharp boundary separates the two antigenic domains and coincides with the boundary between the inner and outer lamellae.

Discussion

Precise ultrastructural localization of specific antigens within the integument of *Drosophila* allows us to demonstrate three major aspects of cuticle protein synthesis and cuticular structure.

The immunochemical localization demonstrates that cuticle proteins detected by our various antisera do indeed reside within the lamellate region of the cuticle. Furthermore, the finding of labeled vesicles supports Locke's conclusions (1976) that at least some cuticle precursors are transported in vesicles.

The differential localization of L-PCPs and H-PCP 82 shows that they are deposited as they are synthesized, first the L-PCPs and then the H-PCPs. Because low levels of anti-LCP antiserum were detected over the inner lamellae we cannot formally rule out the possibility that small amounts of L-PCPs are stored and secreted later. However, binding of this antiserum to the inner lamellae is no greater than random background cytoplasmic binding. This, coupled with the lack



Figures 2 and 3. Fig. 2: A conventional Epon section of the pupal integument 18 h after pupariation showing the major cuticular layers. *OL*, outer lamellae; *IL*, inner lamellae; *AZ*, assembly zone; *E*, epidermis. Bar, 0.5 μm. Fig. 3: Immunolocalization of PCPs on frozen thin sections of 15.5-h pupal integument using anti-PCP. The second antibody was conjugated to 5-nm gold particles. (*a*) Transverse section showing label concentrated over both inner and outer lamellae. Note the relative absence of labeling in the assembly zone. (*b*) An oblique section showing gold-labeled vesicles. Abbreviations as for Fig. 2. Bar, 1.0 μm.

of specificity of the rabbit antiserum (Fig. 1, lane *3*), leads us to conclude that there are few or no L-PCPs in the inner lamellae. Moreover, experiments with MAB 82 unequivocally demonstrate that H-PCP 82 is deposited by apposition and not intussusception. By contrast, intussusception of cuticular components has been demonstrated in other arthropods: *Calpodes ethlius*, (Condoulis and Locke, 1966); *D. melanogaster* larvae, (Kaznowski and Schneiderman, 1980); Elateridae,

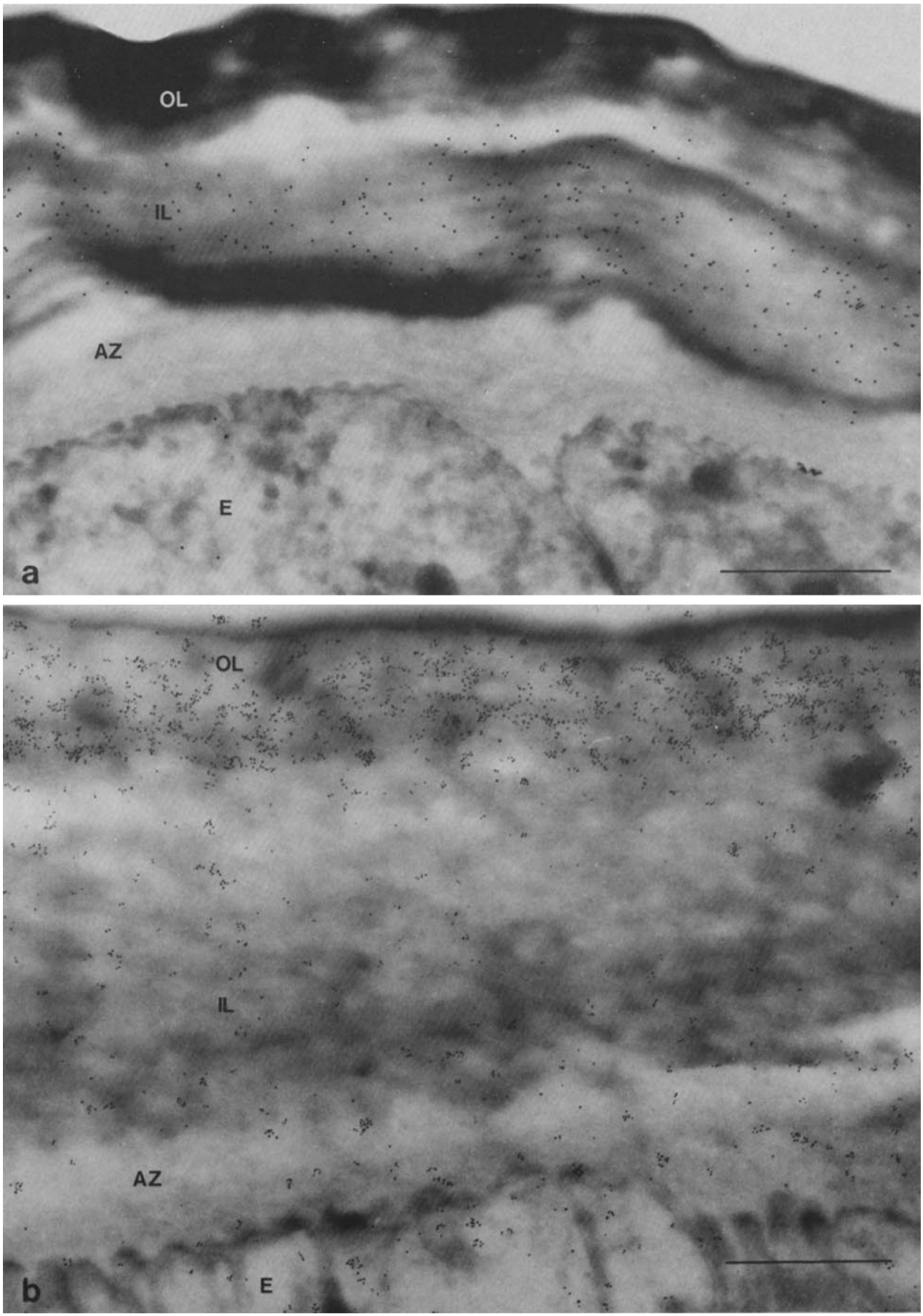


Figure 4. Differential labeling of inner and outer lamellae in the 18-h pupal integument. (*a*) Immunolocalization of PCP 82 using a monoclonal antibody (MAB 82). The second antibody is conjugated to 20-nm gold particles. (*b*) Immunolocalization of L-PCPs using anti-larval cuticle protein antiserum. This antiserum cross-reacts with L-PCPs. The second antibody was conjugated to 5-nm gold particles. *OL*, outer lamellae; *IL*, inner lamellae; *AZ*, assembly zone; *E*, epidermis. Bar, 1.0 μm.

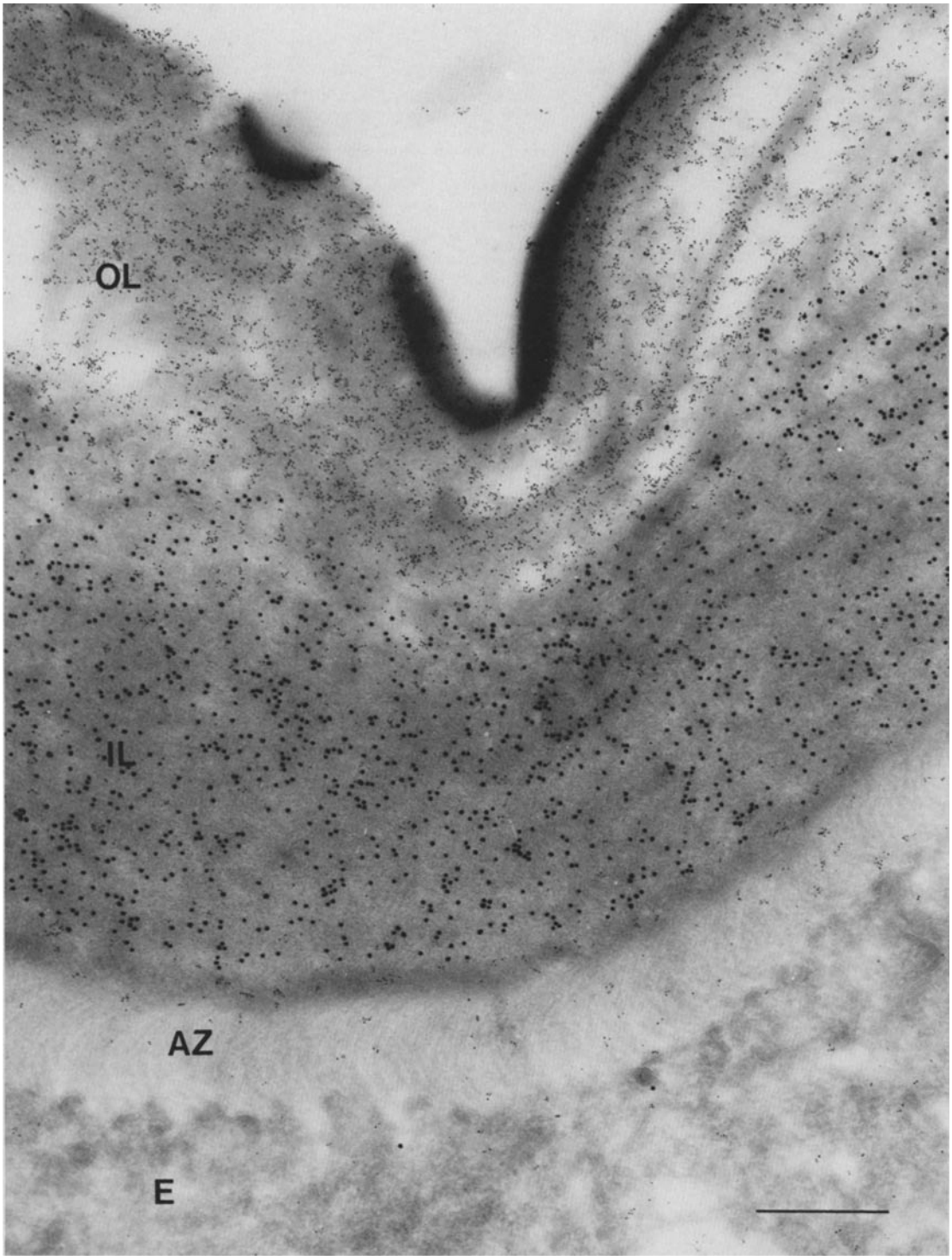


Figure 5. Simultaneous localization of PCP 82 (20-nm gold), and L-PCPs (5-nm gold). The boundary between the two antigenic regions corresponds to the transition from outer to inner lamellae. *OL*, outer lamellae; *IL*, inner lamellae; *AZ*, assembly zone; *E*, epidermis. Bar, 0.5 μm.

(Zacharuck, 1972); *Ioxodes ricinus*, (Lees, 1952); and *Sarcophaga falculata*, (Dennell, 1946).

Furthermore, we demonstrate that the antigenically and morphologically distinct inner and outer lamellae are endowed with distinct sets of proteins. Such differences in cuticular protein composition of inner and outer lamellae have been inferred from studies of cuticle protein synthesis in three other insects: larval *Manduca sexta*, (Wolfgang and Riddiford, 1986); larval *Rhodnius prolixus* (Hillerton, 1978); and *Tenebrio molitor* (Roberts and Willis, 1980*a, b*), as well as in *Drosophila* (Doctor et al., 1985). In all these studies the deposition of inner and outer cuticular regions coincided with qualitative and quantitative changes in cuticle protein synthesis and/or composition. Furthermore, in both pupal *Drosoph-*

ila (Doctor et al., 1985) and larval *Manduca* cuticles (Wolfgang and Riddiford, 1986) the observed changes are evidently brought about by a small rise in 20-hydroxyecdysone titer. Thus, in *Drosophila* pupae a small increase in 20-hydroxyecdysone titer causes a switch in synthesis and deposition from L-PCPs to H-PCPs, (Doctor et al., 1985). This produces an immediate change in the protein composition of the newly deposited cuticle, which may in turn be responsible for the change in lamellar morphology.

The limited binding of any of our antisera with the assembly zone presents an enigma because, as the name implies, this zone is regarded as the site where the chitin and protein self-assemble into lamellae (Neville, 1975). Differential extraction of proteins from the assembly zone during processing is unlikely since vesicles within the epidermis had a positive reaction to the anti-PCP antiserum. We also doubt that antigenic sites of PCPs in the assembly zone are unavailable, because the sites are available in the more compacted lamellar regions. We assume that the filamentous structures of the assembly zone are at least partly protein because pure chitin does not stain with uranyl acetate (Neville, 1975). If the proteins in this region were insoluble in urea they would not have been extracted, and no antibody would have been raised against them. Attempts to isolate and localize assembly zone proteins are under way. Whatever the structure of the assembly zone it appears that the PCPs are either not tightly bound in it or, as has been suggested by Neville (1975), move across this region to become incorporated into overlying lamellae. Thus, we envision that cuticle formation is a two-step process in which a relatively insoluble scaffold, exposed in the assembly zone, becomes embedded in a soluble matrix of protein during the formation of lamellae.

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