The Pupal Cuticle of *Drosophila*: Biphasic Synthesis of Pupal Cuticle Proteins In Vivo and In Vitro in Response to 20-Hydroxyecdysone

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ABSTRACT We investigated the synthesis and localization of *Drosophila* pupal cuticle proteins by immunochemical techniques using both a complex antiserum and monoclonal antibodies. A set of low molecular weight (15,000–25,000) pupal cuticle proteins are synthesized by the imaginal disk epithelium before pupation. After pupation, synthesis of the low molecular weight proteins ceases and a set of unrelated high molecular weight proteins (40,000–82,000) are synthesized and incorporated into the pupal cuticle. Ultrastructural changes in the cuticle deposited before and after pupation correlate with the switch in cuticle protein synthesis. A similar biphasic accumulation of low and high molecular weight pupal cuticle proteins is also seen in imaginal discs cultured in vitro. The low molecular weight pupal cuticle proteins accumulate in response to a pulse of the insect steroid hormone 20-hydroxyecdysone and begin to appear 6 h after the withdrawal of the hormone from the culture medium. The high molecular weight pupal cuticle proteins accumulate later in culture; a second pulse of hormone appears to be necessary for the accumulation of two of these proteins.

A major focus in developmental biology centers on the mechanisms by which batteries of genes that mediate particular developmental events are regulated. The genes encoding the cuticle proteins of *Drosophila* lend themselves to such investigations because their protein products are abundant, their expression is controlled by changes in the titer of the steroid hormone, 20-hydroxyecdysone (20HOE),[1] and their structure and function are accessible to modern genetic analysis.

An insect cuticle is a highly organized extracellular structure secreted by an underlying epidermis. Cuticles consist of an outer waterproofing epicuticle composed of lipids and highly cross-linked proteins, and an inner procuticle composed of chitin and proteins (reviewed in references 2, 20, and 36). Procuticles are composed of helicoidally arranged layers that create a laminate appearance in cross-section when observed by electron microscopy (36). In nonsclerotized cuticles, the procuticle proteins are extractable in solvents such as 7 M urea (14, 16).

Drosophila melanogaster produces five separate cuticles during development: three larval cuticles, one per instar; a pupal cuticle (41), the subject of this article; and an adult cuticle. With the exception of the first and second instar cuticles, each of the cuticles contains a unique set of proteins (5). Most of the proteins of the third instar larval cuticle are encoded in multigene families. For example, one family comprises four genes that are located within 7.9 kilobases of each other on chromosome 2 (51, 52).

The pupal cuticle is particularly useful in studying gene regulation because it can be synthesized in vitro by mass-isolated imaginal disks under defined hormonal conditions. We previously described the deposition of pupal cuticle in imaginal disks cultured in vitro for a total of 18 h with 20HOE present for the first 6 h (13). No procuticle is formed either in the absence or continuous presence of the hormone. Thus, the initial exposure to hormone followed by its removal are essential for procuticle formation. The cuticle formed under these conditions, however, is not complete: it is composed of only a small number of procuticular lamellae containing

[1] *Abbreviations used in this paper*: FITC, fluorescein isothiocyanate; H-PCP, high molecular weight pupal cuticle protein; 20HOE, 20-hydroxyecdysone; IB, immunoprecipitation buffer, 10 mM phosphate, 150 mM NaCl, 0.1% SDS, 0.1% Triton X-100, 0.1% Nonidet P-40, pH 7.4; L-PCP, low molecular weight pupal cuticle protein; PCP, pupal cuticle protein; Staph A, *Staphylococcus aureus*.

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chitin and a subset of the pupal cuticle proteins. In this paper we describe conditions necessary for the synthesis and deposition of a complete pupal cuticle. We show that the cuticle proteins are synthesized and deposited in vivo in two discrete sets: first, a set of low molecular weight proteins accumulates, followed by a set of high molecular weight proteins. The synthesis of the low molecular weight proteins ceases at about the time that accumulation of the high molecular weight proteins begins. This switch in synthesis occurs at pupation (~12 h after the onset of metamorphosis) and correlates with a change in the physical appearance of the cuticular lamellae. We also show that a similar profile of accumulation of cuticle proteins can be obtained in vitro by extending the culture period and modifying the hormonal regimen to mimic the changes in hormone titer that occur in vivo.

MATERIALS AND METHODS

Fly Cultures

Larvae of an Oregon R strain of *Drosophila melanogaster* were cultured en masse (32). Prepupal and pupal stages were timed at 25°C from puparium formation (white prepupa). The prepupal period (12) ends with pupation (eversion of the cephalic complex) ~12 h after puparium formation.

Metabolic Labeling and Isolation of Proteins from Pupal Integuments

The pupal integument (cuticle and underlying epidermis) was dissected in cold Ringer's solution (8) from 10–15 appropriately staged animals and divided into anterior (head, thorax, and appendages) and posterior (abdominal) regions. Whole pupae were used for some experiments instead of dissected integuments. In either case the tissue was extracted for 12–18 h in 7 M urea, 5 mM Tris, pH 8.6, on ice and centrifuged for 10 min in an Eppendorf microfuge. The supernatants from this and other procedures were stored at −20°C.

Dissected pupal integuments were incubated in 30–60 μl Robb's leucine-free culture medium (47) containing 1 μCi [^{3}H]leucine (55 Ci/mmol; Schwarz/Mann, Spring Valley, NY) per μl medium for 30 min at 25°C. At the end of the labeling period, 1.4 ml Robb's medium with [^{1}H]leucine was added and the label was chased for 15 min. The tissue was recovered by centrifugation and extracted for 12–18 h in 100 μl 7 M urea, 5 mM Tris, pH 8.6, on ice.

Metabolic Labeling and Isolation of Proteins from Imaginal Disks Cultured In Vitro

Imaginal disks were isolated en masse from mid- to late-third instar larvae (9) and incubated in Robb's medium supplemented with 1% bovine serum albumin (BSA, Cohn fraction V, Sigma Chemical Co., St. Louis, MO) and 10 μM neomycin sulfate (Sigma Chemical Co.) at 25°C. Typically, 20HOE (1 μg/ml; 2×10^{-6} M) was present at the beginning of culture. After 6 h the hormone was removed by washing the disks three times with 25-ml volumes of Robb's saline (47) at 25°C and the incubation was continued in Robb's medium without hormone. For incubations with continuous hormone, 20HOE was added to the cultures immediately after washout. Metabolic labeling of imaginal disks in vitro was carried out using 10 μCi/ml of ^{3}H]-amino acids (194 mC/mg; catalog #20063, ICN Chemical and Radioisotope Division, Irvine, CA) in Robb's medium that lacked the corresponding amino acids.

After culture, disks were washed two or three times in ice cold Ringer's solution and pelleted. The disc pellet was homogenized in 0.5–1.0 ml 7 M urea, 5 mM Tris, pH 8.6, and the cuticle proteins were extracted as described above.

Isolation of Proteins from Mass Preparations of Pupal Cuticles

Pupal cuticle proteins (PCPs) were prepared from pupal cuticles isolated en masse as previously described (50) to yield milligram quantities of PCPs for use as standards and for immunizations (see below).

PAGE

Electrophoresis was typically done in 15% acrylamide (14.6% acrylamide, 0.4% bis-acrylamide) slab gels under nondenaturing or denaturing (SDS) conditions as previously described (50). Either equal amounts of protein (18) or equal radioactivity (trichloroacetic acid–precipitable counts per minute) were loaded onto gels. Gels were stained with Coomassie Brilliant Blue R-250 (10). Fluorography of dried gels was performed using EnHance (New England Nuclear, Boston, MA) and Kodak SB-5 x-ray film at −70°C.

Immunological Techniques

PRODUCTION OF POLYCLONAL RABBIT ANTI-PCP SERUM: Two New Zealand white rabbits were injected subcutaneously with 0.5 mg PCPs in Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY). Booster injections using Freund's incomplete adjuvant were made 3, 7, and 12 wk after the primary injection. The rabbits were bled 8 d after the final injection. The sera were clarified (4), combined, and stored in 1-ml aliquots at −70°C.

PRODUCTION OF MONOCLONAL ANTIBODIES TO PCPS: BALB/c mice were injected intraperitoneally with 100 μg PCPs in Freund's complete adjuvant. Two intraperitoneal booster injections of 5 μg PCPs in Freund's incomplete adjuvant were given 3 and 8 d later. Spleens were removed 3 d after the final injection. Spleen cells were fused with NS-1 myeloma cells (38) and plated at limiting dilution with or without feeder cells (11). Supernatants from wells containing hybrid cells were screened by an enzyme-linked immunosorbant assay for antibodies against PCPs. Positive lines were expanded and frozen. Two hybridoma lines designated Mab56 and Mab82 because of their specificity for PCP56 and PCP82, respectively, were recloned at limiting dilution. Analysis with immunoglobulin class-specific antisera showed that Mab56 is IgM, and Mab82 is IgG.

PROTEIN GEL BLOTS: Proteins were electrophoretically transferred from gels equilibrated in transfer buffer (50 mM sodium acetate) to cyanogen bromide–activated paper (6). For some analyses, proteins were transferred to nitrocellulose paper (with 24 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol). After transfer, the protein blot was quenched with 1% BSA in 1 M glycine for cyanogen bromide–activated paper blots or 1% BSA in phosphate-buffered saline (PBS; 10 mM phosphate, pH 7.4, 150 mM NaCl) for nitrocellulose blots. Blots were then washed (three times for 10 min each in 250 ml PBS containing 0.1% BSA and 0.1% Nonidet P-40) and incubated with monoclonal antibodies (undiluted culture supernatants) or with complex anti-PCP serum (diluted 1:100 in PBS with 1% BSA). The second antibodies were conjugated to horseradish peroxidase. Goat anti-rabbit Ig/horseradish peroxidase (Bio-Rad Laboratories, Richmond, CA) and goat anti-mouse IgG and IgM/horseradish peroxidase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were diluted 1:750 in PBS with 1% BSA. Bound antibodies were visualized using hydrogen peroxide and 4-chloro-1-napthol (Sigma Chemical Co.) as substrates (19).

ROCKET IMMUNOELECTROPHORESIS: Rocket immunoelectrophoresis was carried out in 1% agarose, 5% anti-PCP serum, 0.5% Triton X-100, 100 mM Tris, pH 8.6. Electrophoresis was at 1 V/cm for 12–16 h at 15°C using an electrode buffer of 100 mM Tris, 0.5% Triton X-100, pH 8.6. Gels were then washed first in 100 mM NaCl for 24 h and then in water for 3–6 h and stained (3).

IMMUNOPRECIPITATION: Samples containing equal trichloroacetic acid–precipitable counts per minute were suspended in 5 ml immunoprecipitation buffer (IB, 10 mM phosphate, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) and clarified by centrifugation at 18,000 *g* for 2 h at 4°C. Samples were preabsorbed with 150 μl of 10% *Staphylococcus aureus* (Staph A) cells (IgG-Sorb; Enzyme Center, Boston, MA) in IB for 30 min at room temperature. The Staph A cells were removed by centrifugation and the supernatant was incubated for 45 min at room temperature in 75 μl preimmune or anti-PCP serum. Immune complexes were then adsorbed for 30 min with 150 μl 10% Staph A cells in IB. The Staph A cells were pelleted and washed three times with 7.5 ml IB. The pellet was transferred with 1.5 ml IB, centrifuged in a microfuge for 5 min, extracted with 100 μl SDS sample buffer at 100°C for 2 min, and clarified by centrifugation (25).

IMMUNOFLUORESCENT MICROSCOPY: For whole-mount preparations disks were fixed in 4% formalin or paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4) for 10 min on ice and washed in PBS. Disks were then allowed to air dry onto slides to make the disks permeable and to attach them to the slides for further processing. As soon as the tissue had dried it was rehydrated with PBS and incubated with antibodies as described below.

For frozen sections, disks or pupae (with the pupal case removed) were fixed for 1–2 h in 4% formalin in 0.1 M sodium cacodylate (pH 7.4) on ice and then kept overnight in 5% sucrose in 0.1 M sodium cacodylate. Frozen sections (4–10 μm) were fixed with 4% buffered formalin onto subbed slides. The sections were incubated in drops of rabbit anti-PCP serum (diluted 1:100 or 1:1,000 in PBS) or undiluted hybridoma cell culture supernatants for 1 h at room temperature. Bound antibodies were reacted with fluorescein isothiocyanate

(FITC)–conjugated secondary antibody or in a two-step process using biotinylated secondary antibody followed by FITC-avidin (Vector Laboratories, Inc., Burlingame, CA). Extensive washes in PBS were carried out after each step. Sections were mounted in Tris-buffered glycerol (pH 8.6) and observed with epifluorescence.

Electron Microscopy

Tissue was fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate from 1 to 24 h at 4°C, postfixed in 2% osmium tetroxide, and left overnight in 70% ethanol to enhance tissue contrast. After a brief treatment in 100% propylene oxide followed by 1 h in a 1:1 mixture of propylene oxide and Polybed (Polysciences Inc., Warrington, PA), the tissue was infiltrated overnight in Polybed. Sections were stained with uranyl acetate and lead citrate.

RESULTS

Studies of Pupal Cuticle Proteins In Vivo

ACCUMULATION OF PCPS: Fig. 1 shows proteins extracted from pupal integuments at different stages after pupariation visualized both by protein gel blot analysis using anti-PCP serum (Fig. 1*A*) and by staining directly with Coomassie Blue (Fig. 1*B*). A Coomassie Blue–stained gel of PCP standards is included for reference (Fig. 1*C*). The cuticle proteins fall into two distinct classes: a low molecular weight class that includes PCPs 15, 19, 21, 22, 23, and 25; and a high molecular weight class that includes PCPs 56 and 82. Of note, the low molecular weight PCPs (L-PCPs) begin to accumulate 8 h after pupariation and are pronounced by 10 h, whereas the high molecular weight PCPs (H-PCPs) begin to appear at 12 h and are pronounced by 14 h after pupariation.

Of the low molecular weight class, PCPs 15, 19, and 21 are major Coomassie Blue–stainable cuticle proteins (Fig. 1*C*) and are readily recognized by the complex antiserum. PCP 19 seems to be sensitive to SDS denaturation and does not appear in Fig. 1*A*; it does appear in gel blot analyses using nondenaturing gels (data not shown). PCP 25 is a major cuticle protein (Fig. 1*C*) but is not well recognized by the antiserum. PCPs 22 and 23 are relatively minor, but immunogenic, cuticle components. Thus, the representatives of the L-PCP class that are present in any given assay vary somewhat according to the assay. It is clear, however, that this group of proteins accumulates at the same time of development (before pupation). Furthermore, many L-PCPs are immunologically related to each other (50). Thus, we will treat the L-PCPs as a group for most of the present analysis.

Of the high molecular weight class, PCPs 56 and 82 are major cuticle proteins and are immunologically distinct from the L-PCPs (50). A polypeptide, which migrates slightly faster than PCP 56, is clearly seen in the protein blot but is only a minor Coomassie Blue–stainable component. Two proteins of ~40,000 mol wt are minor but immunogenic components that begin to accumulate ~14 h after pupariation.

Fig. 1*A* also distinguishes between accumulation of PCPs in the anterior region of the pupa (lanes *A*; head, thorax, and appendages) and the posterior region (lanes *P*; abdomen). Of note, PCP 15 is only found in the anterior pupal integument, an observation previously reported by Chihara et al. (5). Furthermore, the H-PCPs are detected in the anterior integument ~2 h before they are detected in the posterior integument. This lag appears to reflect a general delay in posterior epidermal cell differentiation; it has also been observed in the differentiation of adult cuticular structures (33, 49).

Protein gel blot analyses using monoclonal antibodies

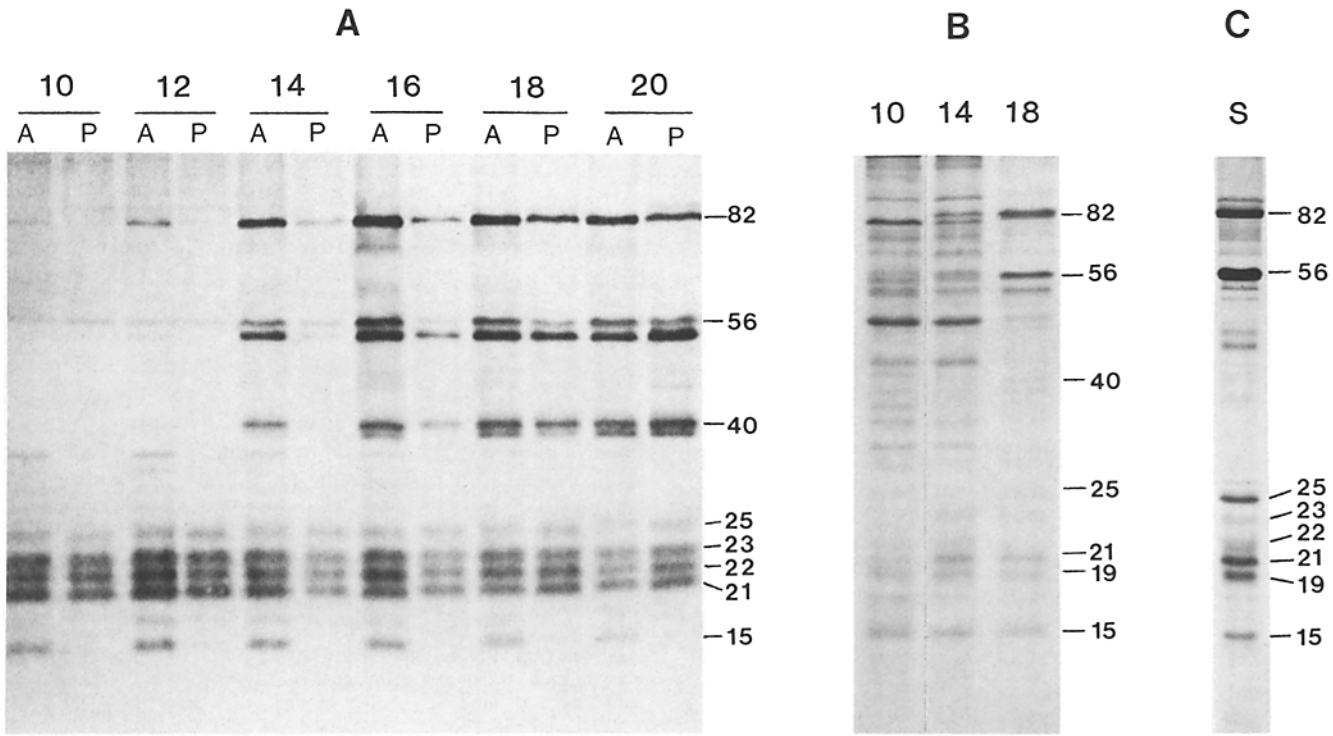

FIGURE 1 Accumulation of PCPs in vivo. (*A*) Protein gel blot of urea-soluble proteins of anterior (*A*) and posterior (*P*) pupal integuments dissected at different times (hours) after pupariation. Proteins (5 μg/lane) were separated by SDS PAGE (15%), electroblotted to cyanogen bromide–activated paper, and incubated with rabbit anti-PCP serum, and immune complexes visualized by immunoperoxidase staining. (*B*) Representative lanes from a comparable Coomassie Blue–stained gel. (*C*) Pupal cuticle standards: SDS-PAGE (15%) of PCPs (30 μg) extracted from mass-prepared pupal cuticles and stained with Coomassie Blue. The major PCPs are indicated.

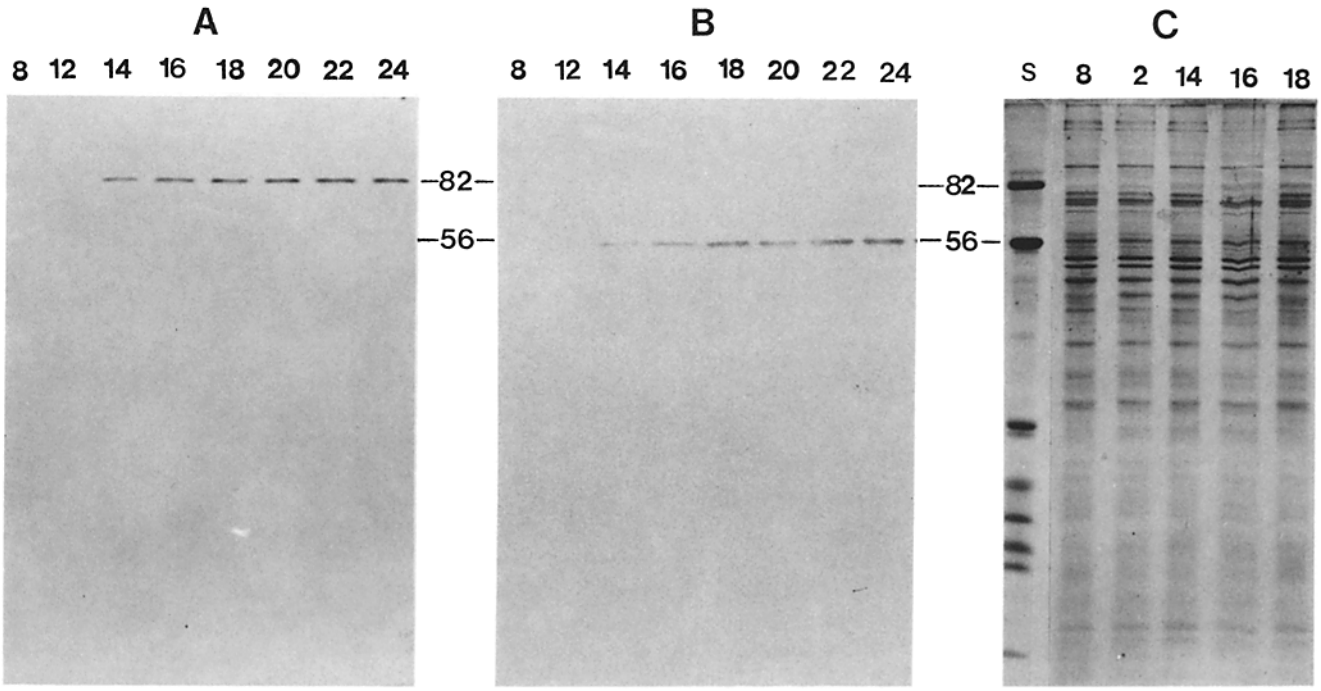

FIGURE 2 Accumulation of PCPs 56 and 82 in vivo. A protein gel blot analysis of urea-soluble proteins extracted from whole pupae at different times (hours) after pupariation. Proteins (20 μg/lane) were separated by SDS PAGE (15%), electroblotted to nitrocellulose, incubated with Mab82 (*A*) or Mab56 (*B*), and visualized by immunoperoxidase staining. (*C*) Representative lanes (20 μg urea-soluble pupal protein/lane) from a comparable Coomassie Blue-stained gel. Lane *S*: 20 μg PCP standards. PCPs 56 and 82 are indicated.

against PCPs 82 and 56 are shown in Fig. 2, *A* and *B*. In agreement with Fig. 1, both of these PCPs are first detected ~14 h after pupariation. In contrast to those in Fig. 1, these blots use proteins extracted from whole pupae rather than from dissected pupal integuments and thus represent a very complex mixture of proteins (Fig. 2*C*). Each monoclonal antibody recognizes a single polypeptide in this mixture of proteins as expected for monospecific antibodies.

ULTRASTRUCTURE OF THE PUPAL CUTICLE: We made electron microscopic observations of the thoracic pupal integument at 8, 10, 12, 14, 16, and 18 h after pupariation. An increase in the number of procuticular lamellae occurs from 10 h (1 or 2 lamellae) to 18 h (up to 20 lamellae) as shown in Fig. 3. At these stages the epithelial surface is studded with microvilli and procuticle fibrils are closely associated with dense plaques at the tips of the microvilli. This surface morphology is often regarded as indicative of active cuticle secretion (27), which suggests that there is continued deposition of procuticular lamellae at least until 18 h after pupariation. This is in contrast to the general view that pupal cutical deposition is complete at pupation (39) and consistent with an observed increase in glucosamine content (presumably in chitin) of the pupal cuticle after pupation (53).

The procuticle of the 18-h pupal cutical can be divided into two morphologically distinct layers: an outer irregular dense layer of three or four poorly defined lamellae and an inner layer of 12–18 regularly arranged lamellae (Fig. 3*C*). Examination of earlier stages indicates that the outer lamellae are deposited before pupation and the inner lamellae after pupation. For example, at 10 h (2 h before pupation) there are two lamellae visible immediately beneath the dense epicuticle (Fig. 3*A*), and by 14 h (2 h after pupation) there are three somewhat dense outer lamellae and three broader less dense inner lamellae (Fig. 3*B*). The difference in density between outer and inner lamellae is more pronounced by 18 h (Fig. 3*C*) and may be due to the partial sclerotization of the outer lamellae as cuticle deposition proceeds. The important point is that the deposition of the outer lamellae coincides with the accumulation of the L-PCPs and the deposition of the inner lamellae coincides with the accumulation of H-PCPs.

LOCALIZATION OF PCPS BY INDIRECT IMMUNOFLUORESCENT MICROSCOPY: We used the monoclonal antibodies and rabbit anti-PCP serum to localize the PCPs to the pupal integument using indirect immunofluorescent microscopy on frozen sections. The complex antiserum stains the pupal cuticle very intensely and the basal lamina lightly. Basal lamina staining was eliminated by preabsorbing the antiserum with intact, mass-isolated, third instar imaginal disks (basal lamina is external at this stage of disk development). When this preabsorbed serum is used, the resultant staining is cuticle specific (Fig. 4, *A* and *B*). Intense staining with this antiserum is seen from 10 h after pupariation onward. In controls using preimmune serum, only the faint autofluorescence of the epicuticle is observed (Fig. 4*C*).

The staining seen with Mab82 is intense and completely cuticle specific (Fig. 4, *D–F*). At 10 h after pupariation no staining could be seen with this antibody. Staining is first detected in limited areas of the 12-h pupal cuticle. Intense staining in the thoracic region develops by 14 h and persists through 74 h after pupariation (the latest stage examined). From 48 to 74 h, adult cuticle is present but shows no reaction with Mab82. Staining of the abdominal pupal cuticle with Mab82 lags several hours behind that of the thorax. In regions where the cuticle could be seen in cross-section, fluorescent label is observed throughout most of the thickness of the cuticle (Fig. 4*F*). In the region indicated by the arrow one

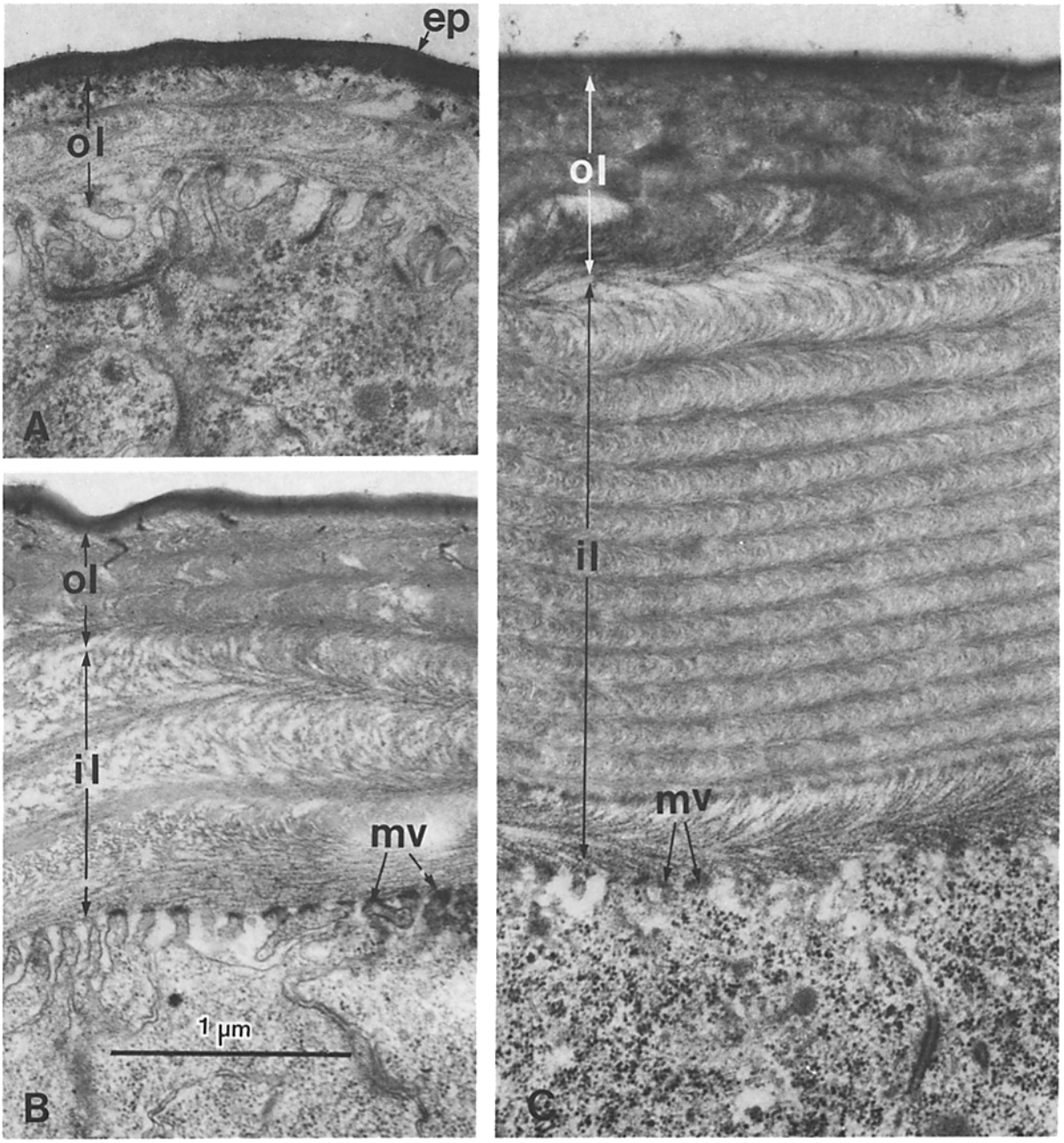

FIGURE 3 Electron micrographs of thoracic pupal integument at (*A*) 10, (*B*) 14, and (*C*) 18 h after pupariation. *ol*, outer lamellae of procuticle; *il*, inner lamellae; *mv*, microvilli with dense tips; *ep*, epicuticle, × 36,000.

can distinguish a thin outer fluorescent band believed to be the autofluorescent epicuticle, then a narrow unstained region that may correspond to the outer lamellae, and finally a broad band which stains specifically with Mab82. This suggests that PCP82 is structurally associated with the inner lamellae.

Indirect immunofluorescent analysis with Mab56 shows some faint general staining of the pupal cuticle at 12 h after pupariation that becomes intense by 24 h. Staining with Mab56 appears to occur throughout most of the thickness of the cuticle (data not shown).

METABOLIC LABELING OF PCPS IN PUPAL INTEGUMENT: Thus far we have presented data concerning the accumulation and localization of the PCPs in the pupal integument (cuticle and underlying epidermis). We have also examined the protein synthetic activity of the anterior and posterior pupal integument at the same stages (Fig. 5). Nondenaturing gels (Fig. 5*A*) are usually used to separate the L-PCPs. Note that PCPs 21, 19, and 15 are heavily labeled at 10 h (and to a lesser extent at 12 h) and are the major synthetic product in this molecular weight range. After this time no incorporation into these L-PCPs can be seen. SDS gels are necessary to separate the H-PCPs (Fig. 5*B*). Incorporation of [^{3}H]leucine into PCP 82 was first detected at about 12 h (Fig. 5*B*) and into PCP 56 at ~14 h after pupariation (Fig. 5, *A* and *B*).

Immunoprecipitations were carried out with anti-PCP serum (Fig. 6) to confirm that these newly synthesized polypeptides were PCPs and to clarify the picture of PCP synthesis. Consistent with the results presented in Fig. 5, L-PCPs are actively synthesized at 10 h after pupariation and not there-

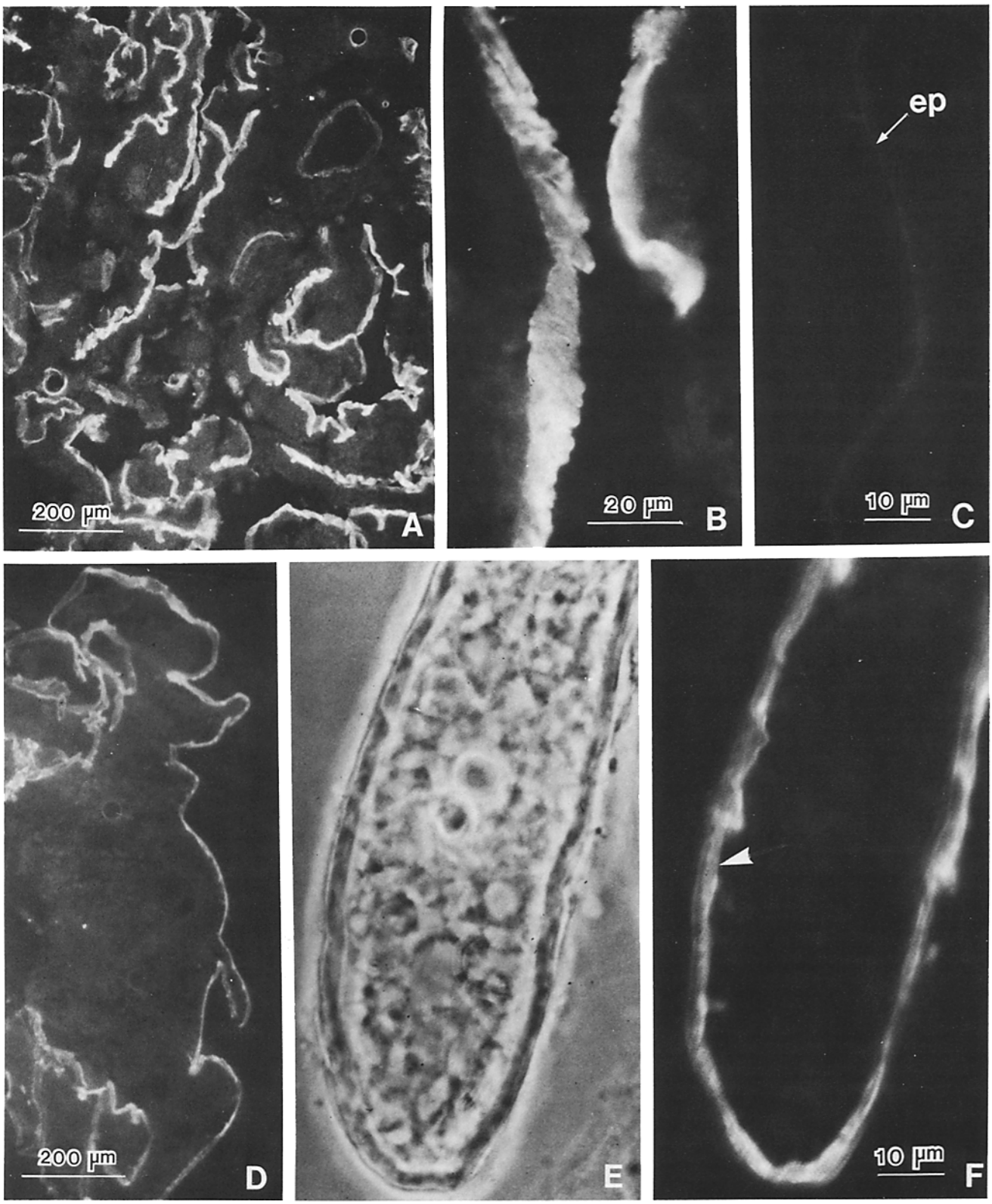

FIGURE 4 Localization of PCPs in pupal thoracic integument by indirect immunofluorescent staining of frozen sections. (*A* and *B*) 10-μm section of thoracic integuments from 10-h pupae incubated first with anti-PCP serum and then with FITC-conjugated anti-rabbit serum. Only the cuticle, which is seen here in surface (not cross-sectional) view is stained. *A*, × 100. B, × 900. (*C*) Control incubated with preimmune serum. The faint signal is due to autofluorescence of the epicuticle (*ep*). × 1,250. (*D* and *F*) Transverse 4-μm section of the thoracic region of an 18-h pupa stained first with Mab82 then with biotinylated-anti-mouse serum and FITC-avidin. (*E*) Phase-contrast image of *F*. Arrows indicate a region of the cuticle where three layers can be distinguished in cross-section: the autofluorescent epicuticle, a nonfluorescent outer region, and a fluorescent inner region. *D*, × 100. E and F, × 1,250.

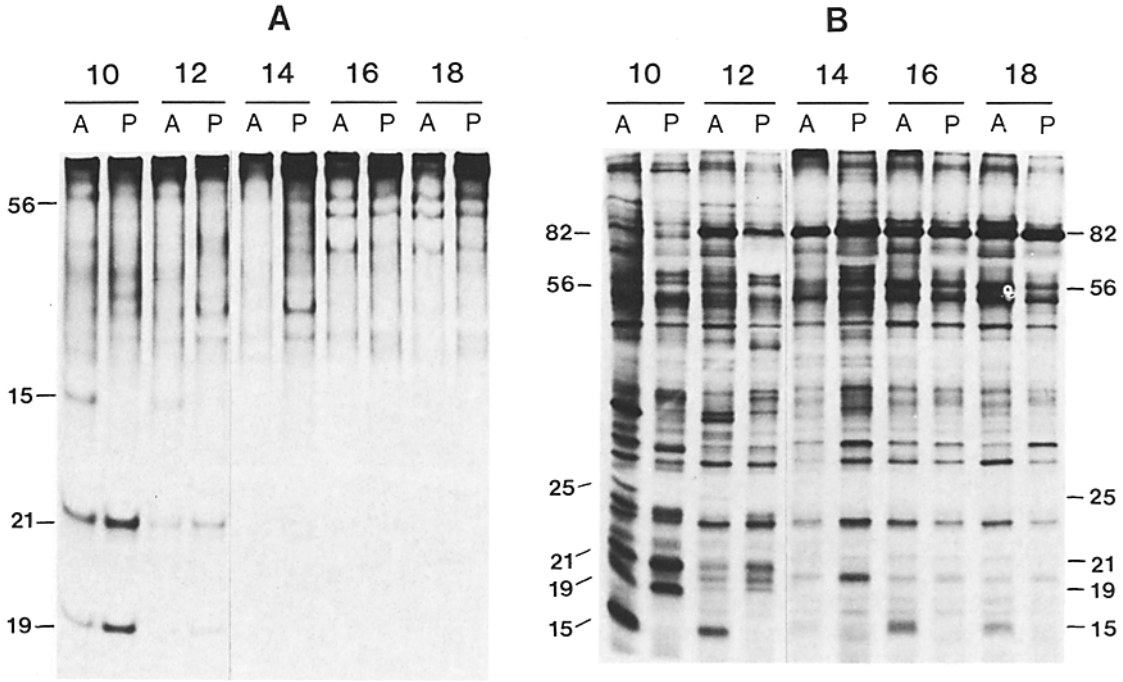

FIGURE 5 Protein synthesis in anterior (*A*) and posterior (*P*) pupal integuments dissected at different stages (hours) after pupariation and incubated for 30 min with [^{3}H]leucine. Fluorograms of urea-soluble ^{3}H-proteins (20,000 trichloroacetic acid-precipitable cpm per lane) separated by nondenaturing (*A*) or SDS PAGE (*B*). Exposures were for 6 d at −70°C. The positions of the major PCPs are indicated.

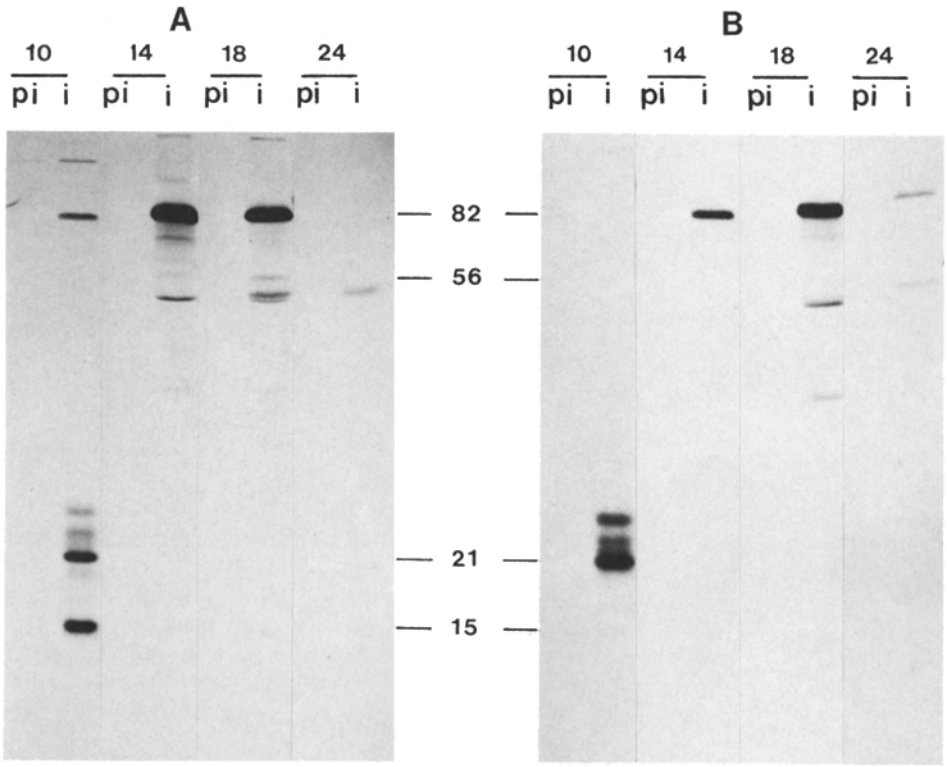

FIGURE 6 Immunoprecipitation of proteins synthesized by anterior (*A*) and posterior (*B*) pupal integument at different stages (hours) after pupariation. Proteins (metabolically labeled and extracted as in Fig. 5) were immunoprecipitated with anti-PCP serum and separated by SDS PAGE. 600,000 trichloroacetic acid-precipitable cpm were reacted with preimmune serum (*pi*) or immune serum (*i*), followed by absorption with Staph A cells. Exposures were for 10 d at −70°C. The positions of the major PCPs are indicated.

after. Furthermore the H-PCPs are synthesized largely after L-PCP synthesis has ceased. H-PCP 82 is the major immunoprecipitable product at 14 and 18 h in both anterior and posterior integument. PCP 56 apparently formed only a weak double band even though it incorporates [^{3}H]leucine (see Fig. 5*B*). This may be due to interference from the heavy immunoglobulin chain, which has a similar electrophoretic mobility. Again, note that PCP 15 is only immunoprecipitated from the anterior region (compare Figs. 1*A* and 5*A*), whereas L-PCPs 21, 22, and 23 are detected in both anterior and posterior hypoderm.

The presence of a precipitin band for H-PCP 82 at 10 h in the anterior integument reguires further explanation. This is the only instance in which we have evidence for the presence

of PCP 82 before pupation. This may be a result of poor staging of the pupae used for this time point. However, bear in mind that this is the most sensitive of the assays used and that there may indeed be limited localized synthesis of H-PCP 82 before pupation corresponding to the localized bands of this protein seen after 24 h in vitro (see Fig. 11).

Taken together these observations demonstrate that an abrupt switch from L-PCP synthesis to H-PCP synthesis occurs in vivo at about the time of pupation.

The Accumulation of Pupal Cuticle Proteins in Imaginal Disks In Vitro

ACCUMULATION AND LOCALIZATION OF L-PCPS IN IMAGINAL DISKS: During the course of this and previous work (13) on the synthesis and accumulation of the PCPs by mass-isolated imaginal disks cultured in vitro, we routinely used an 18-h in vitro culture period with 1 μg 20HOE/ml (2×10^{-6} M) present for the first 6 h. For convenience we refer to this hormone regimen as a hormone pulse. Under these conditions, imaginal disks secrete a pupal cuticle and synthesize both chitin and the L-PCPs. Fig. 7 shows the time course of appearance of the L-PCPs by rocket immunoelectrophoresis in response to the hormone pulse regimen in imaginal disks cultured in vitro. Two points are notable. First, immunoreactive material is detected in disks incubated with a hormone pulse but not in disks cultured either in the absence or the continuous presence (1 μg/ml) of hormone. Second, the earliest detection of immunoreactive material occurs 7 h after hormone withdrawal. Protein gel blots show that only the L-PCPs were synthesized under these conditions (see Fig. 10, lane *1*). These results corroborate earlier work (13) that showed that the synthesis of PCPs 15, 19, and 21, and the deposition of procuticular lamellae by mass-isolated discs in vitro occur in response to a hormone pulse.

The cellular localization of the PCPs in cultured disks was determined by indirect immunofluorescent microscopy on frozen sections (Fig. 8). In disks cultured for 18 h with the continuous presence of hormone (0.1 μg/ml) no staining above background is seen (Fig. 8*A*). In disks incubated under hormone pulse conditions, fluorescent staining is localized at the apical surface, presumably in the pupal cuticle (Fig. 8, *B* and *C*). These results are consistent with the immunochemical observations above and indicate that L-PCPs not only accumulate under hormone pulse conditions but are also secreted into the pupal cuticle.

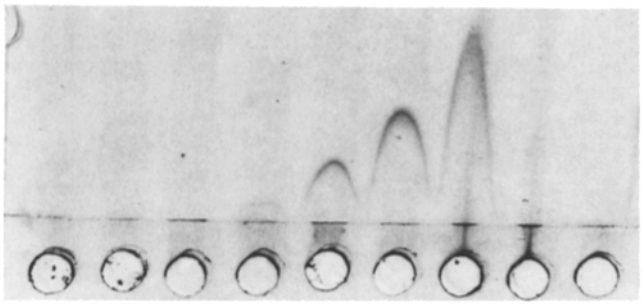

FIGURE 7 Rocket immunoelectrophoretic analysis of PCP accumulation in imaginal disks cultured in vitro. Urea-soluble protein (30 μg/well) from disks cultured for different times (hours) with 20HOE (1 μg/ml; 2×10^{-6} M) present for only the first 6 h was electrophoresed into agarose containing anti-PCP serum. Wells marked – and + contained urea-soluble proteins from disks cultured for 18 h in the absence (–) and continuous presence (+) of 20HOE (1 μg/ml). Gel was stained with Coomassie Blue.

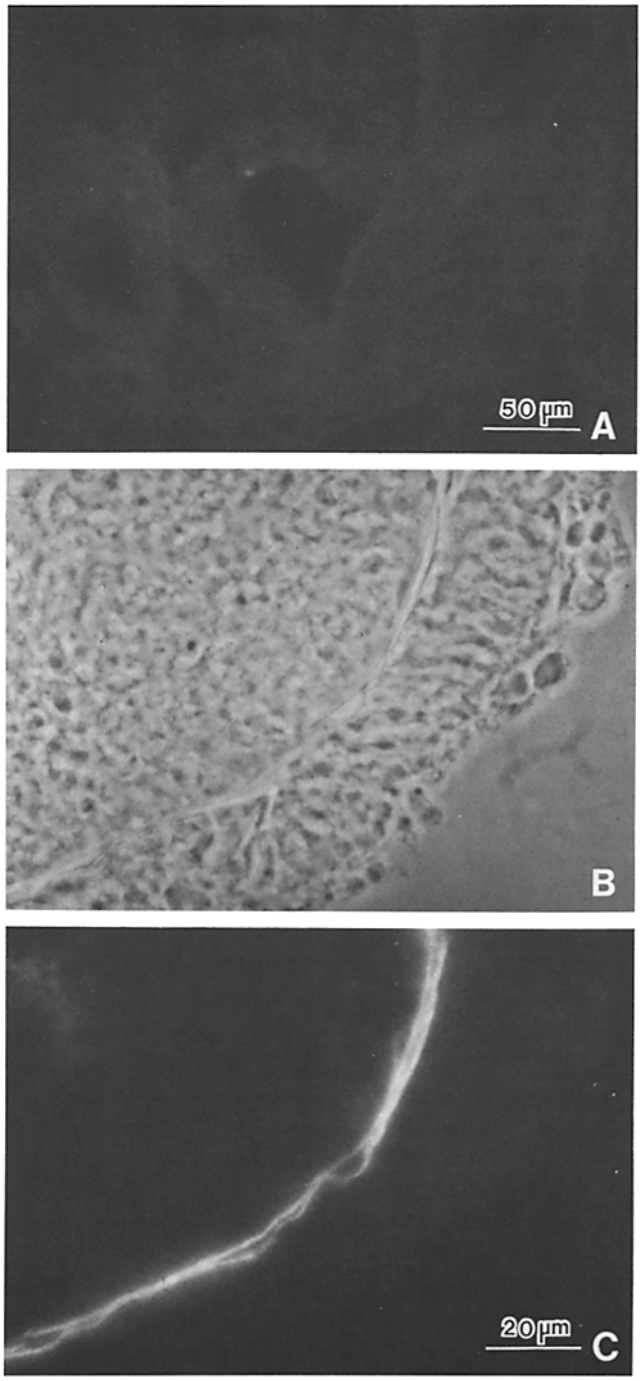

FIGURE 8 Indirect immunofluorescent analysis with anti-PCP serum of frozen sections of imaginal disks cultured in vitro. (*A*) Disks incubated for 18 h in continuous 20HOE (0.1 μg/ml). No procuticle is present and no specific staining is observed. × 320. Phase-contrast micrograph (*B*) and fluorescence microgram (*C*) of a disk incubated with 20HOE (1 μg/ml) present for the first 6 h. The double line of fluorescence in *C* indicates the presence of PCPs in the pupal procuticle at the apical surface of the folded disk epithelium. × 800.

EFFECT OF READDITION OF HORMONE ON L-PCP SYNTHESIS: The effects of the readdition of 20HOE to culture

medium on L-PCP synthesis and accumulation are shown in Fig. 9. Disks were incubated for 21 h with the standard hormone pulse from 0–6 h. Hormone was added back to half of the disks between 14 and 17.5 h (8–11.5 h after hormone withdrawal) and ^{3}H-amino acids were added to all disks for a 3-h period between 18 and 21 h. The readdition of hormone in this incubation was intended to mimic a reported increase in 20HOE titer that occurs in vivo during the late prepupal period (~10–12 h after pupariation; 17, 26). The fluorogram shown in Fig. 9 demonstrates that the incorporation of ^{3}H-amino acids into PCPs 15, 19, and 21 that normally occurs under hormone pulse conditions is inhibited with the readdition of hormone.

ACCUMULATION OF PCP56 AND PCP82 IN IMAGINAL DISKS IN VITRO: The H-PCPs were not detected in disks cultured in vitro with the standard 18-h hormone pulse regimen. The data presented above indicate that PCPs 56 and 82 appear in vivo considerably later than the L-PCPs. We therefore increased the length of our in vitro culture from 18 to 24 h with hormone present for the initial 6 h. In addition, some disks were cultured as above with 0.1 μg 20HOE/ml present between 14 and 18 h of culture. The protein gel blot in Fig. 10 shows that PCP 82 is detected after 24 h with the readdition of hormone between 14 and 18 h (Fig. 10, lane *4*). In addition, an intense band appears at ~40,000 and probably corresponds to the 40K polypeptides identified in Fig. 1. These observations suggest that the accumulation of PCP 82 and the 40,000-mol-wt protein were dependent on the readdition of 20HOE. On the other hand, PCP 56 was present at 24 h both with and without the readdition of hormone but apparently in higher amounts with the readdition of hormone (Fig. 10, lanes *3* and *4*).

PCPs 56 and 82 were localized in imaginal disks by indirect immunofluorescent microscopy using monoclonal antibodies Mab56 and Mab82, respectively. Imaginal disks were incubated under the conditions described above. Neither Mab56 or Mab82 stains disks incubated in vitro for only 18 h. Mab56 stains disks that had been incubated for 24 h either with or without the readdition of hormone; this result is consistent with the accumulation of PCP56 shown in the protein gel blot analysis of Fig. 10. The staining was uniformly distributed over the sheetlike pupal cuticle (Fig. 11*A*).

The immunofluorescent pattern obtained with Mab82 after 24 h of culture differs strikingly depending on whether or not

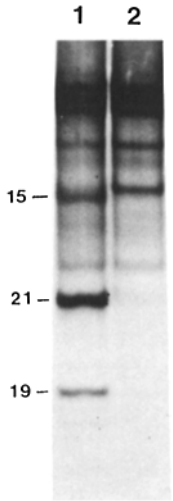

FIGURE 9 Incorporation of ^{3}H-amino acids into PCPs by imaginal disks cultured in vitro. Fluorogram of urea-soluble ^{3}H-proteins from imaginal disks cultured for 21 h separated by nondenaturing PAGE (100,000 trichloroacetic acid-precipitable cpm per lane). Discs were cultured with 20HOE (1 μg/ml) for only the first 6 h (*1*) or with 20HOE for both the first 6 h and (0.1 μg 20HOE/ml) between 14 and 17.5 h (*2*). ^{3}H-amino acids were present in the culture medium between 18 and 21 h. Exposure was for 20 d at −70°C. The positions of the major PCPs are indicated.

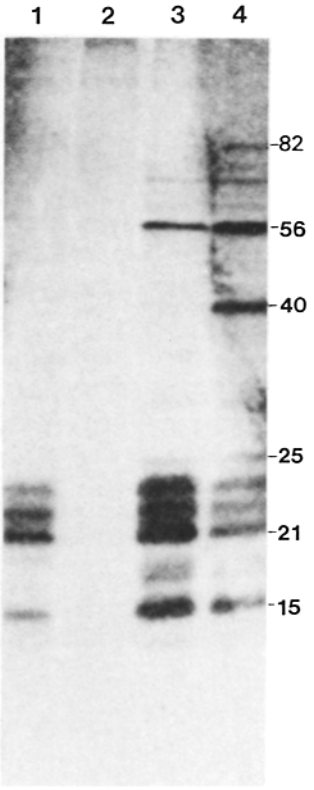

FIGURE 10 Accumulation of PCPs in imaginal disks cultured in vitro. Protein gel blot analysis using anti-PCP serum of urea-soluble proteins from imaginal disks. Protein (30 μg/lane) was separated by SDS PAGE (15%), electroblotted to cyanogen bromide-activated paper, incubated with rabbit anti-PCP serum, and visualized by immunoperoxidase staining. Disks were cultured for 18 h with 20HOE (1 μg/ml) present for the first 6 h (lane *1*) or continuously (lane *2*). Disks were cultured for 24 h with 20HOE (1 μg/ml) present for the first 6 h and without (lane *3*) and with (lane *4*) the readdition of 20HOE (0.1 μg/ml) between 14 and 18 h.

hormone was added back to the culture medium. Disks incubated for 24 h with hormone present for only the first 6 h of culture had fluorescent staining limited to a small region of the total pupal cuticle deposited in these disks (Fig. 11, *B* and *C*), whereas disks incubated under the same conditions but with 20HOE also present for 4 h between 14 and 18 h of culture stain with Mab82 over the entire area of pupal cuticle deposited in vitro (Fig. 11*D*). The limited staining observed without the readdition of hormone was restricted to a very precise double band of pupal cuticle on leg imaginal disks (Fig. 11, *B* and *C*). This stained region is localized to the margin of the thorax-forming region of the disk that ultimately fuses with corresponding regions of other disks.

DISCUSSION

We have demonstrated that a set of L-PCPs is synthesized and accumulates in the pupal cuticle in vivo before pupation, and a set of H-PCPs is synthesized and accumulates in the pupal integument primarily after pupation. Furthermore, the cuticular lamellae deposited before pupation (outer lamellae) and after pupation (inner lamellae) are ultrastructurally distinct. Immunofluorescent observations using complex antiserum against the full complement of PCPs specifically localizes these polypeptides in the pupal cuticle. In addition, analysis with a PCP 82–specific monoclonal antibody suggests that this polypeptide may be confined to the inner lamellae of the pupal procuticle. These observations provide strong evidence that the L-PCPs are structural components of the outer lamellae of the pupal procuticle, deposited before pupation and the H-PCPs are structural components of the inner lamellae deposited after pupation. This conclusion has now been confirmed by immunogold labeling of ultrathin frozen sections (Wolfgang, W. J., and D. Fristrom, manuscript in preparation).

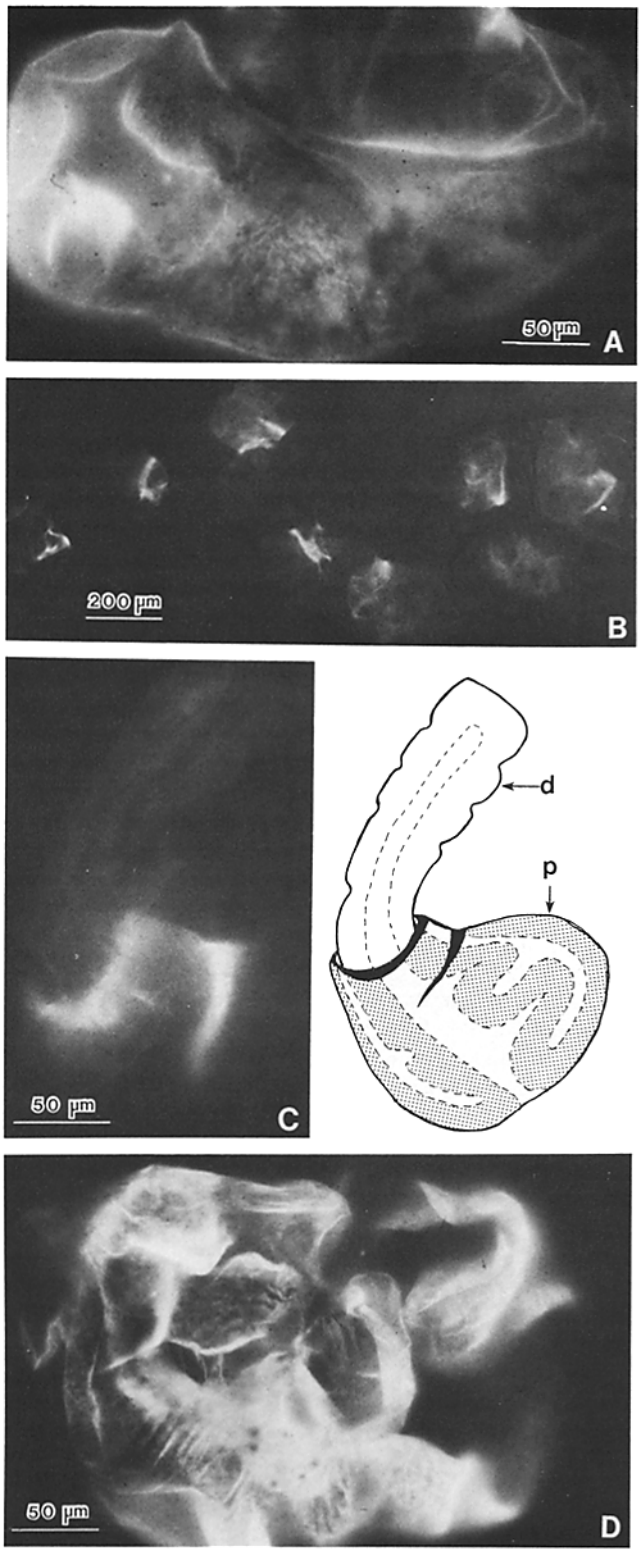

FIGURE 11 Indirect immunofluorescent analysis of imaginal disks cultured in vitro using anti-PCP monoclonal antibodies. Disks were incubated for 24 h with an initial 6-h hormone pulse with or without readdition of 20HOE (0.1 μg/ml) between 14 and 18 h. (*A*) Disk cultured without hormone addback and incubated with Mab56. × 360. (*B* and *C*) Disks cultured without hormone-addback and incubated with Mab82. *B*, × 80. *C*, × 400. Two fluorescent bands are consistently observed on the proximal edge of the disk as indicated in the diagram. Note that under these incubation conditions, evagination is not complete and the proximal (thorax-forming) region is deflected back over the distal appendage-forming region. (*D*) Disk cultured with hormone-addback and incubated with Mab82. Fluorescent staining covers the entire pupal cuticle. × 240. Only the proximal region (stippled area in diagram) forms pupal cuticle in vitro.

Differential synthesis of cuticle proteins can also be tissue related (55). In the results presented here it is clear that PCP15 is confined to the anterior integument (see also reference 5). The anterior pupal cuticle is deposited by the imaginal disks whereas the posterior (abdominal) cuticle is deposited mainly by the larval epidermis with contributions from the genital disk and imaginal histoblasts (48). Thus, the accumulation of PCP15 in disk-derived tissue may result from the tissue-specific synthesis of this polypeptide.

Differential temporal synthesis and/or accumulation of cuticle proteins during the deposition of a cuticle has been observed for various insects including *Tenebrio molitor* (45, 46), *Rhodnius prolixus* (21), and *Manduca sexta* (23, 24, 56). In *Drosophila*, the differential synthesis of proteins during the formation of adult integumental structures (33), as well as the differential accumulation of adult (49) and third instar cuticle proteins (Kimbrell, D., and J. Fristrom, manuscript in preparation), coincides with changes in levels of transcripts encoding the polypeptides. Work is in progress in our laboratory to isolate the genes encoding the PCPs and thus permit an analysis of regulation at the level of transcription.

Results from a number of in vitro systems (1, 29, 34, 35, 37) including *Drosophila* imaginal disks (7, 15, 28, 30, 31) demonstrate that the deposition of insect cuticles is controlled by 20HOE. In this study we have used various immunochemical techniques to examine the accumulation and localization of pupal cuticle proteins in imaginal disks cultured in vitro in response to different hormone regimens. These results shed light on the role of changes in the levels of 20HOE on pupal procuticle formation. Specifically, it appears that two sequential pulses of 20HOE are required to produce the major procuticle components identified to date:

(*a*) Incubation of disks with an initial hormone pulse (1 μg 20HOE/ml; 2×10^{-6} M) results in the accumulation of the L-PCPs; the accumulation of these proteins is first detected 7 h after hormone withdrawal. These results confirm a previous report from this laboratory demonstrating that the synthesis of both chitin and PCPs 15, 19, and 21 occurs in response to a hormone pulse (13). Furthermore, the induction of DOPA-decarboxylase (an enzyme involved in the formation of compounds used in sclerotization of cuticles) and the accumulation of DOPA decarboxylase mRNA (Clark, W., J. Doctor, J. Fristrom, and R. Hodgetts, manuscript in preparation) occurs only when disks are cultured with a hormone pulse. The onset of the synthesis of all of these macromolecular cuticular components is ~6 h after hormone withdrawal. The hormone pulse regimen approximates changes in the titer of 20HOE during the early stages of *Drosophila* metamorphosis; 20HOE reaches a concentration of $\sim 10^{-7}$ to 10^{-6} M (reviewed in reference 44) for 6 h before pupariation and then drops to intermolt levels during the mid-prepupal period (22). The deposition of the pupal procuticle in vivo begins during the mid-prepupal period, 8–9 h after pupariation (54).

(*b*) The readdition of 20HOE to the culture medium 8–9 h after hormone withdrawal has two distinct effects:

(i) The readdition of 20HOE results in a substantial increase in the accumulation of PCP 82, which is reflected in a change

in the distribution of this polypeptide in disks. An increase in the accumulation of a polypeptide with an apparent molecular weight of 40,000 is also readily observed.

(ii) The hormone addback also inhibits the incorporation of precursor into the L-PCPs. We previously showed that the readdition of hormone at 10 h after withdrawal has no detected effect on chitin synthesis in vitro, whereas the readdition of 20HOE to cultures only 6 h after withdrawal results in the inhibition of chitin synthesis (13). It is apparent from the data presented here that cuticle deposition continues in vivo after L-PCP synthesis has stopped; biochemical evidence also indicates that chitin synthesis continues after pupation (53). Thus, the previously observed lack of inhibition of in vitro chitin synthesis with an addback of hormone at 10 h after hormone withdrawal more closely approximates the in vivo situation.

Hormone was added back to the culture medium in an attempt to mimic changes in the titer of 20HOE observed in vivo. Two studies provide direct evidence for a transient increase in 20HOE titer at approximately the time of pupation (17, 26). Indirect evidence for an increase in 20HOE titer at this time includes the observation that the ecdysone-secreting ring gland is active (40) and that several ecdysone-stimulated salivary gland puffs are induced at this time (43). This transient increase in the titer of 20HOE near the time of pupation appears to be important in the regulation of PCP synthesis. By duplicating the rise in titer at pupation with an addback of hormone in vitro we can duplicate some of the in vivo changes including turning on or increasing the synthesis of some of the H-PCPs and turning off the synthesis of the L-PCPs.

An interesting parallel with disks is seen in the in vitro culture of *Drosophila* salivary glands (42, 43). The same sequence of polytene chromosome puffs observed in vivo during the late larval and prepupal periods is observed in vitro when salivary glands are incubated with a hormone pulse protocol similar to the regimen used in this report; the removal of 20HOE from the medium is required for the in vitro induction of the prepupal puffs (42). Furthermore, it is necessary to add 20HOE back to the culture medium in order to observe the late prepupal puffs in vitro (43). These similarities may reflect common regulatory strategies in the response of both salivary glands and imaginal disks to 20HOE. Moreover, they demonstrate a primary role of changes in the titer of 20HOE in the temporal ordering of developmental events, namely puff sequences and sequences of cuticle protein accumulation, during metamorphosis in *Drosophila*.

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