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The Procuticle of *Drosophila:* Heterogeneity of Urea-Soluble Proteins†

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ABSTRACT: Proteins, soluble in 7 M urea, 4 M guanidine hydrochloride, or 2% sodium dodecyl sulfate, have been extracted from untanned larval cuticles of *Drosophila melanogaster.* A major protein fraction, apparent molecular weight 8000–10 000, is resolved into eight different components (five major, three minor) by gradient gel electrophoresis under nondenaturing conditions. Proteins extracted in 7 M urea have been resolved by diethylaminoethylcellulose chromatography into five fractions, three of which are greatly enriched for electrophoretically homogeneous proteins. The five fractions have different amino acid compositions. Electrophoretic variants involving four of the five major proteins have been obtained. Preliminary genetic analysis indicates that at least three of the five proteins are specified by separate structural genes.

The cuticle of insects is an extracellular structure which serves functionally as both skin and skeleton. The cuticle is composed of an outer epicuticle and an inner procuticle. The epicuticle, as seen by transmission electron microscopy, is a trilayered structure with two electron dense layers separated by an electron transparent layer. The procuticle is composed of helicoidally arranged fibers and has a laminated appearance in sectioned material. The fibers in the procuticle are composed of chitin, a β-(1,4)-linked unbranched polymer of *N*-acetylglucosamine, and protein. In sclerotized cuticle the proteins are cross-linked, while in unsclerotized cuticles there is little cross-linking. X-ray crystallographic analyses of unsclerotized cuticles suggest that in some cases the proteins exist in repeated structures associated with three or four chitobiose residues [see Rudall (1976) for review]. Extraction with solvents such as 7 M urea eliminates the repeating structure and releases proteins into the solvent (Rudall and Kenchington, 1973). Indeed, 7 M urea has been found to release proteins from all kinds of unsclerotized cuticles which have been investigated (Hackman, 1972, 1974a, 1976). The extracted proteins bind to chitin when exposed to the polysaccharide in the absence of urea at pH 7 (Hackman, 1976). For most insects studied, the urea-soluble protein fraction is heterogeneous, as judged by $NaDodSO_4$[1]–gel electrophoresis and isoelectric focusing (Hackman, 1972, 1974a, 1976). Comparisons among several genera demonstrate that these proteins are not conserved during evolution.

The cuticle is deposited by an underlying layer of epidermal cells in response to ecdysteroids.[2] Cuticle deposition by epidermal tissues cultured in vitro with ecdysteroids has been demonstrated by workers in several laboratories [for review, see Marks and Sowa (1976)], including imaginal disks of *Drosophila* (Mandaron, 1976; Fristrom, unpublished observation). Current evidence indicates that ecdysteroids act on target tissues by interacting with the chromatin and modulating transcription (Ashburner et al., 1973; Fragoulis and Sekeris, 1975; Yund and Fristrom, 1975a,b) in a manner similar to that proposed for vertebrate steroid hormones (reviewed by Gorski and Gannon, 1976; Yamamoto and Alberts, 1976).

The different urea-soluble cuticle proteins may be derivatives, variants, or multimers of a single polypeptide (Hackman, 1975, 1976) or involve polypeptides with different primary structures. If the last is the case, cuticle formation in insects, particularly in *Drosophila* where genetic analysis is possible, may serve as an excellent system for the study of coordinate gene regulation in eukaryotes. We report in this paper characteristics of procuticle proteins isolated from unsclerotized larval cuticles of *D. melanogaster.* We conclude that at least five different polypeptides specified by three or more genes are present.

Experimental Procedures

An *Ore. RC* strain of *D. melanogaster* maintained in mass culture in the Genetics Research Laboratories was used in

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[1] Abbreviations used are: 7 MUT, 7 M urea, 0.005 M Tris, pH 8.6; Tris, tris(hydroxymethyl)aminomethane; PUT, phenylthiourea; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; binding buffer, 0.005 M NaCl, 0.005 M Tris, pH 7.0; CP, cuticle protein.

[2] The term ecdysteroid refers to any member of a family of steroids with molting hormone activity.

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these studies. Larvae were grown at 19 °C in plastic boxes on heavily yeasted medium and were recovered by washing with water during the late third instar.

Preparation of Larval Cuticles. Cuticles were prepared by two different methods. (1) Larvae were ground in medium G-2 using the automated dissector of Zweidler and Cohen (1971). Larval carcasses were recovered on nylon screening and were then transferred to 4 volumes of cold (4 °C) *Drosophila* Ringer's solution (Ephrussi and Beadle, 1936) containing 1% (v/v) mercaptoethanol and saturated with PTU, an inhibitor of tyrosinase. The carcasses, about 70 g wet weight, were then blended in a Waring blender at top speed for 2–3 min. The resulting cuticles were recovered on nylon screening. The cuticles were washed with water and then resuspended in 5 volumes of cold 0.5 M Tris-HCl, pH 7.0, containing 1% mercaptoethanol and saturated with PTU. The cuticles were again blended for 2–3 min as above and then recovered on the nylon screening. The recovered cuticles were washed with water, blotted dry, and stored at −70 °C or used immediately. (2) The second procedure was identical to the first except the larvae were ground directly in the blender. The resulting cuticle preparations are free of contaminating tissues when viewed at 50× with a dissecting microscope.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using a bovine serum albumin standard.

Protein Extraction. (1) $NaDodSO_4$. Cuticles were exposed to 2% $NaDodSO_4$ (w/v) at room temperature for 15 min with stirring, recovered on nylon screening, and then extracted in 2% $NaDodSO_4$ for 6 h at 60 °C. Extracted proteins were precipitated in the cold by the addition of 4 volumes of acetone. The precipitate was recovered by centrifugation and washed twice with acetone. (2) Urea. Frozen cuticles were thawed in the cold for 3 h with stirring in 4 volumes of 7 MUT containing 1% mercaptoethanol and saturated with PTU. The extract was filtered through glass wool and then poured over DEAE-cellulose (Whatman DE-52, 50-mL packed volume) equilibrated with 7 MUT. Cuticle proteins were displaced with 0.1 M NaCl in 7 MUT and stored frozen at −70 °C. (3) Guanidine. Unfrozen cuticles are extracted for 1 h in 4 volumes of cold 4 M guanidine hydrochloride. The extract was filtered through glass wool and stored frozen at −70 °C. The above extracts are respectively referred to as crude $NaDodSO_4$, urea, and guanidine cuticle protein extracts.

To extract proteins from single larval cuticles, a larva was placed on a microscope slide in Ringer's solution containing 1% mercaptoethanol and saturated with PTU. The rear of the larva was removed with watchmakers forceps, and the larva was then turned inside out on a dissecting needle. Viscera were scraped off and the carcass was transferred to a drop of 0.5 M Tris-HCl, pH 7, containing 1% mercaptoethanol and saturated with PTU. The carcass was scraped with a dissecting needle to remove body wall musculature and the underlying epidermis. The scraped cuticle was rinsed two additional times in 0.5 M Tris-HCl, pH 7.0, immersed in distilled water, and then blotted dry on a tissue. The cuticle was then placed on parafilm and extracted at room temperature for 15 min with 10 μL of 7 MUT containing sufficient Bromophenol blue to be used as tracking dye during electrophoresis. In practice, 10–20 separate cuticles were dissected, placed on parafilm in a prescribed order, and simultaneously extracted with 7 MUT. Extracts were then frozen or used immediately for electrophoresis.

Gel Electrophoresis. Vertical gel electrophoresis was carried out using the following gel systems:

(1) Urea-Gel Electrophoresis. Slab acrylamide gels (7%) were formed with dimensions of 10.5 × 13.6 × 0.08 cm in 0.075 M Tris-citrate, pH 8.6, containing 7 M urea as described by Hill et al. (1971). The gels were overlaid with 0.075 M Tris-citrate, pH 8.6, containing 3 M urea. The upper and lower buffer reservoirs were filled with 0.3 M boric acid adjusted to pH 8.6 with NaOH. A few drops of Bromophenol blue were added to the upper reservoir. Cuticle protein samples (5–20 μL) were applied to the gel in 7 MUT. Electrophoresis, carried out at room temperature with constant current, was begun at 10 mA and increased to 20 mA when the Bromophenol blue boundary from the reservoir buffer entered the gel.

(2) Standard Slab Gels. Slab acrylamide gels were prepared and run as described above, except that urea was omitted from the gel-forming solution.

(3) Gradient Slab Gels. Preformed, 3–28% acrylamide slab gels (8 × 8 cm) were obtained from Gradient Pty. Ltd., Lane Cove, N.S.W., Australia. Upper and lower buffer chambers were filled with 0.1 M Tris-borate, pH 8.9. Samples (5–10 μL) were applied in 7 MUT. Electrophoresid was carried out at 400 V in a chamber holding four gels.

(4) $NaDodSO_4$-Gel Electrophoresis. Tube gels (75 × 2 mm) containing 10% acrylamide were formed in 0.1 M sodium phosphate, pH 7.2, and 0.1% $NaDodSO_4$. Crude extracts were dialyzed against 0.2% $NaDodSO_4$, 0.01 M phosphate, pH 7.2, 0.14 M mercaptoethanol, 10% (v/v) glycerol before loading onto the gels. Gels were run at 60 V/tube until the tracking dye was 15 mm from the bottom. Ovalbumin, 43 kdaltons; yeast alcohol dehydrogenase, 37 kdaltons; carbonic anhydrase, 29 kdaltons; soybean trypsin inhibitor, 21 kdaltons; myoglobin, 17.2 kdaltons; RNase, 13.7 kdaltons; cytochrome *c*, 11.7 kdaltons; and unreduced insulin, 5.6 kdaltons, were used as molecular weight standards.

All gels were stained with Coomassie Blue R.

DEAE-Cellulose Chromatography. Crude urea cuticle protein (50–100 OD_{280nm} units; for purified cuticle proteins 1 OD_{280nm} = 0.6–1 mg of protein) in 50–200 mL of 7 MUT was applied to a DEAE-cellulose column (0.9 × 10 cm) equilibrated with 7 MUT. Proteins were eluted using gradients from 0.03 to 0.07 M and 0.07 to 0.1 M NaCl in 7 MUT using a 250-mL mixing volume and flow rate of 25 mL/h. Chromatography was carried out at 2–4 °C. The OD_{280nm} was monitored continuously or was determined on 5-mL fractions. Positions of different cuticle proteins in the column eluate were determined by electrophoresis of undiluted 20-μL samples. Fractions were kept frozen as much as possible to minimize formation of carbamyl derivatives due to any traces of cyanate in the urea solutions.

Affinity Chromatography. Affinity chromatography was performed on columns of purified chitin. Chitin (Sigma, technical grade) was washed with 4 M guanidine hydrochloride until there was no OD_{280nm} detected in the washes. The chitin was introduced into a column (0.9 × 10 cm) and washed overnight with binding buffer. Crude urea cuticle proteins were exhaustively dialyzed in the cold against repeated changes of binding buffer containing 1% (v/v) mercaptoethanol. The mercaptoethanol was omitted during the last dialysis. The dialyzed protein (1.5 mL) was introduced onto the column and allowed to stand for 3 h at room temperature. The chitin column was then washed successively with 12-mL volumes of binding buffer, 0.5 M Tris-HCl, pH 7.0, or 0.5 M NaCl, 7 MUT, and in some experiments involving noncuticle proteins with 4 M guanidine hydrochloride. One-milliliter fractions were recovered. The binding of myoglobin (Sigma, type I), ovalbumin (Nutritional Biochemicals, crystalline), and egg-white lysozyme (Sigma, type I) was also studied.

Amino Acid Compositions. Protein samples were dialyzed in the cold against 0.05 M sodium phosphate, pH 12,

and then against 0.005 M NaOH. Samples were lyophilized, dissolved in 0.5 mL of constant-boiling HCl (Pierce sequanol grade), 0.01% mercaptoethanol, and 0.01% phenol, and then hydrolyzed for 24 h at 110 °C. The hydrolysates were dried overnight in a vacuum desiccator over NaOH and then dissolved in 1.2 mL of 0.01 N HCl containing 5 nM norleucine. Amino acid analyses were performed on a single-column Jeolco JLC-6AH instrument fitted with high-sensitivity 10-mm flow cells.

Microscopy. Dissected, everted cuticles, extracted or unextracted in 7 MUT, were cut into pieces in distilled water and fixed for 2 h in 1% glutaraldahyde, 1% OsO_4 in 0.05 M sodium cacodylate, pH 7.4. The material was dehydrated in ethanol, passed through two changes of propylene oxide, and then placed for 1 h in a 1:1 mixture of propylene oxide and Epon, and finally embedded in Epon. Thick sections (1 μm) were lightly stained in 1% toluene blue and examined in phase contrast. Thin sections were stained with uranyl acetate and bismuth subnitrate and examined in a Siemen's Elmiskop I.

Recovery of Electrophoretic Variants in Natural Populations. Wild populations of *D. melanogaster* were sampled at eight different wineries in the Hunter Valley in New South Wales. Flies were netted from swarms found mainly around leaking casks. Single inseminated females, at least 50 from each population, were placed into separate vials. Single third instar larvae were recovered from 441 different vials. Proteins were extracted from dissected cuticles and subjected to electrophoresis on gradient gels. Gels were screened for varients of the five major cuticle proteins.

Identification of Chromosomes Carrying Variant Cuticle Protein Alleles. To determine the chromosome carrying the gene specifying a particular cuticle protein variant, a laboratory balancer stock was used as follows: *In (1)*sc^{SIL} sc^{8R} $+^{S}$, sc^{S1} sc^{8} w^{a} *B; In(2LR)SM1,al*^{2} *Cy cn*^{2} *sp*^{2}*/In(2LR)bw*^{Vl}, ds^{33k} dp^{6} bw^{Vl}; *In(3LR)Ubx*^{130},*Ubx*^{130}e^{s}*/In(3LR)C,Sb;* spa^{pol}.

Cy, bw^{Vl} (subsequently called *Pm*), *Ubx,* and *Sb* are dominant mutants lethal in homozygotes marking the second and third chromosomes. *B* marks the X chromosome and the recessive trait spa^{pol} marks the tiny fourth chromosome. With the exception of the small fourth chromosome, each chromosome in the stock is identified by the presence of a dominant marker, allowing the determination of the genetic composition of the progeny produced in the crosses described below. These chromosomes also carry inversions to prevent recombination. (See Lindsley and Grell, 1968, for a full description of the chromosomes and mutants used.)

The following crossing scheme was used:

(1) Ten wild-type virgin ♀♀ from a vial in which an electrophoretic variant had been identified were individually mated to 2–4 balancer ♂♂. Single third instar larvae from each vial were tested for electrophoretic variants of cuticle proteins. Adult ♂♂ were recovered from the vials in which larvae with variant cuticle proteins were found. These ♂♂ were +; *+/Cy* or *+/Pm; +/Sb* or *+/Ubx;* and $+/spa^{pol}$. Since no putative X-linked hemizygous variants were found in the initial screen, nor in the progeny of the initial cross to the balancer stock, it was concluded and subsequently verified that none of the major protein variants was carried on the X chromosome.

(2) Ten progeny ♂♂ from the above cross were individually mated to 4–6 balancer ♀♀. Progeny larvae were tested for electrophoretic variants of the cuticle proteins. Appropriate progeny from vials found to contain variants were mated as described in 3A–C.

(3) Progeny of the following type were mated: (A) $w^{a}B$; *+/Pm; Sb/Ubx* ♂♂ × $+/w^{a}B$; *+/Pm; Sb/Ubx* ♀♀; (*B*) $w^{a}B$; *?/Cy; +/Sb* ♂♂ × $+/w^{a}B$; *Pm/Cy, +Sb* ♀♀; (*C*) $w^{a}B$; *?/Cy; +/Ubx* ♂♂ × $+/w^{a}B$; *Pm/Cy, +/Ubx* ♀♀.

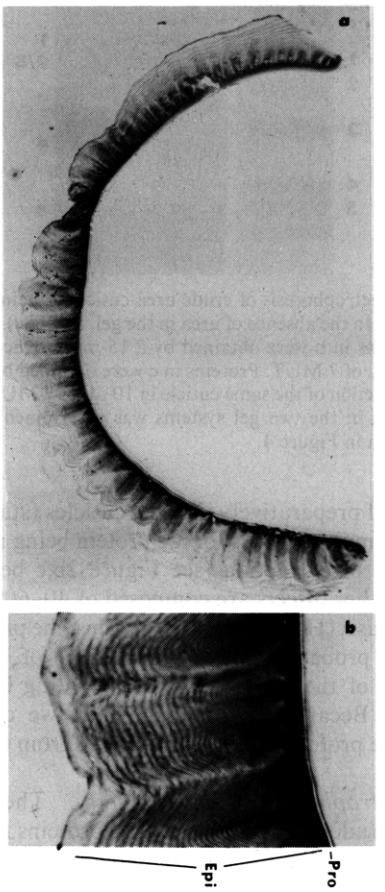

FIGURE 1: Light micrographs of sectioned, dissected cuticles. (a) ×100 (b) ×400. Epi: epicuticle; Pro: procuticle.

Since the recessive trait w^{a} is epistatic to *Pm,* it is not possible to identify *Pm* in the presence of w^{a}. Thus, the ♂♂ in crosses B and C may be *+/Cy* or *Pm/Cy.* However, non-*Cy* ♂♂ in cross A must be *+/Pm.*

Larval progeny were recovered and the cuticle proteins were subjected to electrophoresis. If the variant allele were carried on the second chromosome, electrophoretic variants will be present in cross A and may be present in crosses B and C because of the indeterminant genetic constitution of the ♂♂ in these crosses. If the variant were carried on the third chromosome, the electrophoretic variant will be absent in cross A but will be present in crosses B and C. Progeny homozygous for the different variant alleles will also be produced.

Results

Characteristics of Purified Cuticles. Both dissected and preparatively isolated larval cuticles have the same appearance when viewed by light microscopy as fresh material or in sectioned material after fixation by light and electron microscopy. No underlying epidermal cells are found (Figure 1), although remnants of the cell membranes may be still present. The cuticles have the characteristic appearance with a thin outer epicuticle and a thick inner laminated procuticle. Extraction with 7 MUT does not alter the basic appearance of the cuticles with the epicuticle and procuticle layers still being clearly identifiable. In both extracted and unextracted cuticles, the procuticle layer constitutes about 95% of the material seen in section.

Properties of Extracted Cuticle Proteins. (*a*) *Yield.* Overnight extraction with 7 MUT releases 0.22 mg of protein/mg

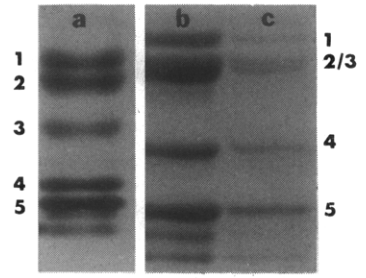

FIGURE 2: Electrophoresis of crude urea cuticle proteins in slab acrylamide gels. (a) In the absence of urea in the gel. (b and c) In the presence of urea. Proteins in b were obtained by a 15-min extraction of a single cuticle in 10 μL of 7 MUT. Proteins in c were obtained by an additional overnight extraction of the same cuticle in 10 μL of 7 MUT. The identity of the proteins in the two gel systems was determined using purified fractions shown in Figure 4.

dry weight of preparatively isolated cuticles (standard deviation ±0.013 mg) with most of the protein being released after only 15 min of extraction (see Figure 2b,c below). Larval cuticles of other insects are composed of 40–60% chitin on a dry weight basis (Hackman, 1974b). Thus, the protein released by 7 MUT probably constitutes 40–50% of the nonchitin components of the larval cuticles (including both epi- and procuticle). Because of the high yield, we conclude that urea-soluble proteins are mainly derived from the procuticle region.

(*b*) *Electrophoretic Heterogeneity.* The results of subjecting crude, urea-soluble cuticle proteins to electrophoresis in 7% acrylamide gels are depicted in Figure 2a. An identical electrophoretic pattern is obtained using crude $NaDodSO_4$- and guanidine-extracted cuticle proteins. In all three extracts, based on intensity of staining, five major proteins are resolved. Minor bands are also routinely resolved, two of which migrate more slowly and two of which migrate more rapidly than the major proteins. The slow-migrating proteins will not be considered further in this report. Since $NaDodSO_4$ binds strongly to proteins and alters electrophoretic mobility, the indistinguishable electrophoretic mobilities of $NaDodSO_4$- and urea-extracted proteins are somewhat surprising. However, we presume that the precipitation of the $NaDodSO_4$-extracted proteins with acetone results in the removal of the $NaDodSO_4$ from the proteins. Using gradient gels, five major bands are again seen, but three rapidly migrating minor proteins are resolved (see below, Figure 6). The proteins are designated CP-1 through CP-8 as a function of increasing electrophoretic mobility. On occasion, a minor band migrating immediately below CP-2 is also seen using gradient gels but is not included in the above number assignments. In the presence of urea (Figure 2b) the electrophoretic pattern is similar to that seen in Figure 2a, but CP-3 is found to migrate only slightly faster than CP-2, giving the appearance of only four major bands. Also, CP-4 migrates, relative to CP-5, more slowly in urea than in its absence. The rapid release of the soluble proteins by 7 MUT is also demonstrated. Figure 2b shows the proteins released by a 15-min extraction of a single cuticle in 10 μL of 7 MUT. Figure 2c shows the proteins released from the same cuticle by an additional overnight extraction in 10 μm of 7 MUT. Cuticle proteins have also been extracted in 7 MUT from unsclerotized cuticles formed by imaginal epidermal cells in the prepupal and pupal periods. In these extracts, proteins which migrate slightly slower than CP-5 have been seen as well as proteins with similar, but distinguishable, migration to CP-1 and CP-2.

(*c*) *Affinity Chromatography.* It is likely that proteins located in the procuticle are directly associated with chitin (Rudall and Kenchington, 1973). Assuming that the urea-soluble proteins were bound to chitin in the procuticle, these proteins may bind to chitin upon renaturation. Urea-soluble proteins extracted from cuticles have been found to bind to chitin at pH 7 (Hackman, 1976). We would require, however, that if the binding to chitin in vitro is the same as the binding in situ that the conditions for release of bound proteins, in vitro and in situ, be the same. Table I demonstrates two experiments in which cuticle proteins were renatured and bound to chitin as described under Experimental Procedures. The proteins bind quantitatively to the polysaccharide. Washing with excess binding buffer, 0.5 M Tris, pH 7, or 0.5 M NaCl removes little of the bound proteins (5–10%). However, the bound cuticle proteins are quantitatively displaced from the chitin by 7 MUT (90–95% recovery). Thus, the conditions for release of the cuticle proteins from chitin in vitro are equivalent to those for the release of the proteins from cuticles. This observation is consistent with the view that the proteins are bound to chitin in situ. For comparison (data not shown), the binding of myoglobin, ovalbumin, and lysozyme to chitin was also investigated. Chitin is a substrate for the last of these proteins, lysozyme. Also, lysozyme is known to bind strongly to chitin (estimated binding constant = 10^5–10^6 M^{-1}) (Cherkasov and Kravchenko, 1970). Under the conditions used in this study, we find that myoglobin does not bind to chitin. Ovalbumin binds to chitin, but is removed by 0.5 M NaCl. Lysozyme binds tightly and is removed by 4 M guanidine hydrochloride and not by 7 MUT. Thus, none of the tested proteins have binding properties identical to the cuticle proteins.

TABLE I: Affinity Chromatography of Renatured Cuticle Proteins.[a]

	expt 1		expt 2	
	OD_{280nm}	%	OD_{280nm}	%
loaded onto col	4.75	100	3.7	100
in bind. buff wash	0	0	0	0
in 0.5 M Tris wash	0.24	5.3		
in 0.5 M NaCl wash			0.39	10.6
in 7 MUT wash	4.2	93.3	3.3	89.4
total recov	4.44	98.6	3.69	99.7

[a] The 0.5 M NaCl was buffered at pH 7 with 5 mM Tris. Details are in Experimental Procedures.

Column Chromatography. Crude urea cuticle proteins were chromatographed on DEAE-cellulose as described under Experimental Procedures. The elution pattern of the proteins is depicted in Figure 3. For further purification of different cuticle proteins, selected fractions from three or four chromatographic separations were pooled, diluted with an equal volume of 7 MUT, and rechromatographed on DEAE-cellulose as described below.

CP-1 and CP-2. Fractions 32–40 and their equivalents were recovered and rechromatographed using a 0.04–0.07 M NaCl gradient in 7 MUT. Peak fractions were pooled, rebound to DEAE-cellulose, and displaced with 0.2 M NaCl in 7 MUT. The resulting material was dialyzed against distilled water, lyophilized, and stored at −70 °C (yield ca. 22 mg).

CP-3. Fractions 15–19 and their equivalents were pooled and rechromatographed using a gradient of 0.03–0.07 M NaCl in 7 M NaCl in 7 MUT. The resulting material was dialyzed against distilled water, lyophilized, and stored at −70 °C (yield ca. 9 mg).

CP-4. Fractions 45–50 and their equivalents were pooled and rechromatographed using a 0.04–0.07 M NaCl gradient in 7 MUT. The leading edge of the resulting peak, containing

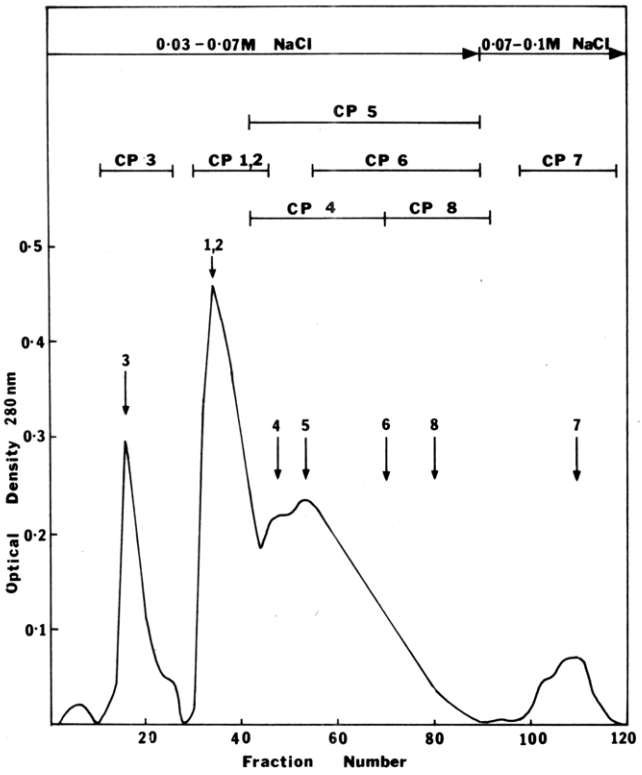

FIGURE 3: Elution profile of crude urea cuticle proteins from DEAE-cellulose column in 7 MUT. Details are in Experimental Procedures. The arrows indicate the peak fractions for the different proteins; the brackets indicate the range over which the different proteins eluted.

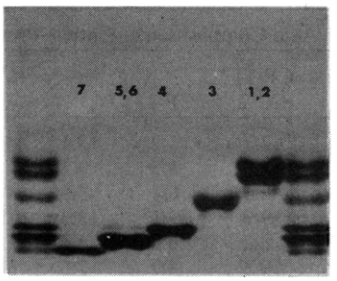

FIGURE 4: Electrophoresis of purified cuticle proteins in 7% slab acrylamide gel. Crude urea cuticle proteins are included for reference. Migration is from top to bottom.

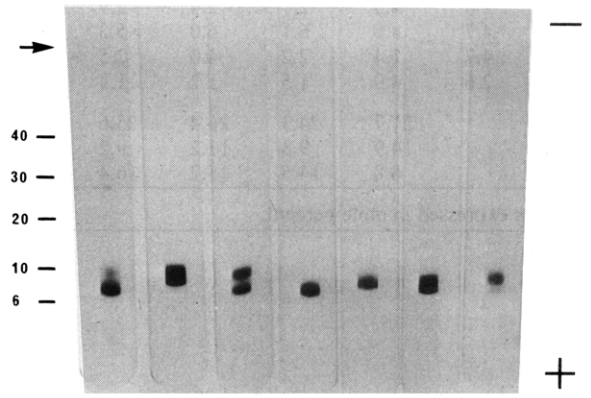

FIGURE 5: $NaDodSO_4$–gel electrophoresis of purified cuticle proteins. Samples from left to right are: (1) crude $NaDodSO_4$ cuticle protein, (2) CP-1/CP-2, (3) crude urea cuticle protein, (4) CP-3, (5) CP-4, (6) CP-5/CP-6, (7) CP-7. The arrow indicates the origin. Molecular weights are given in kilodaltons.

CP-4 and CP-5, was recovered and rebound to DEAE-cellulose. The column was then washed with 0.005 M Tris, pH 8.6 (no urea), and the proteins were eluted with a 0.04–0.07 M NaCl gradient. The resulting CP-4 fractions [separated from (CP-5)] were bound to DEAE-cellulose and displaced with 0.2 M NaCl. The resulting material was frozen at −70 °C (yield ca. 2.5 OD_{280nm}).

CP-5 and CP-6. Fractions 54–70 and their equivalents were recovered and rechromatographed using a 0.04–0.07 M NaCl gradient in 7 MUT. Fractions from the trailing side of the peak were recovered and rechromatographed under the same conditions. Fractions from the trailing side of the peak were again recovered, bound to DEAE-cellulose and displaced with 0.2 M NaCl in 7 MUT. The resulting material was dialyzed against distilled water, lyophilized, and stored at −70 °C (yield ca. 6 mg).

CP-7. Fractions 105–115 and their equivalents were recovered and rechromatographed on DEAE-cellulose using a 0.05–0.1 M NaCl gradient in 7 MUT. Peak fractions were recovered, bound to DEAE-cellulose, and displaced with 0.3 M NaCl in 7 MUT. The resulting material was dialyzed against distilled water, lyophilized, and stored at −70 °C (yield 3 mg).

Electrophoretic Analysis of Purified Fractions. Samples of the purified fractions were subjected to electrophoresis on 7% acrylamide gels in the absence of urea (Figure 4) and appear to be 95% free of contaminants from other fractions. Similar homogeneity was found when the purified fractions were subjected to electrophoresis on urea gels and gradient gels. Samples from the purified fractions were also subjected to $NaDodSO_4$ gel electrophoresis along with crude $NaDodSO_4$ and urea extracts (Figure 5). The crude urea- and $NaDodSO_4$-extracted proteins are fractionated on $NaDodSO_4$ gels into two bands with apparent molecular weights of 8000 and 10 000. The 10 000 molecular weight fraction is reduced in the $NaDodSO_4$ extract because of the preextraction in $NaDodSO_4$ at room temperature. The slower migrating material is CP-1 and CP-2. The remaining cuticle proteins have apparent molecular weights of about 8000. A minor secondary band in the CP-7 fraction is revealed during $NaDodSO_4$ gel electrophoresis.

Amino Acid Composition of Purified Fractions. The amino acid compositions of the different protein fractions are presented in Table II. The compositions of the different fractions are not identical, which indicates that at least some of the proteins, as extracted, have different polypeptide sequences and are not derivatives of a common polypeptide (e.g., containing different numbers of sugar residues) or genetic variants of a common polypeptide. No amino sugars were detected in the hydrolysate. The ratio of acidic to basic amino acids increases with increasing electrophoretic mobility, as would be expected for a series of different polypeptides with similar molecular weights. These results do not eliminate the possibility that some of the polypeptides are derived from a common precursor by proteolysis or share a common region.

Recovery of Cuticle Protein Variants in Wild Populations. In an initial screen of 441 larvae (representing 882 haploid genomes) from eight different locations, four types of variants affecting the electrophoretic mobility of the major proteins on gradient gels were identified (Figure 6).

CP-1. Two types of electrophoretic variants which affect only the mobility of CP-1 were found [a slow and a fast CP-1 (not shown)]. The variants were initially identified in heterozygotes. The gels contained an extra band and there was a visible reduction in the staining intensity of the band at the normal CP-1 position. When homozygotes of the variants were

TABLE II: Amino Acid Composition of Cuticle Proteins.[a]

	crude urea	CP-1/ CP-2	CP-3	CP-4	CP-5/ CP-6	CP-7
Asp	12.1	12.7	15.2	13.1	12.0	12.0
Thr	5.0	2.0	2.5	3.5	8.5	4.7
Ser	6.6	8.4	6.8	5.8	6.9	5.6
Glu	12.0	9.0	9.1	13.3	13.6	16.6
Pro	5.5	8.2	6.6	6.6	4.3	4.5
Gly	9.9	9.3	8.7	7.5	10.1	11.3
Ala	10.3	11.9	9.8	9.2	9.9	11.8
Val	9.2	9.0	11.8	12.0	10.6	10.3
Ile	5.1	5.4	7.6	5.0	3.3	4.4
Leu	4.6	4.0	5.9	6.3	4.2	2.8
Tyr	4.8	3.0	3.9	4.1	4.5	5.5
Phe	2.8	2.3	2.1	2.4	2.9	4.2
Lys	4.7	8.6	6.1	6.0	5.3	2.6
His	4.7	1.4	2.2	4.0	2.5	1.4
Arg	2.6	4.9	1.5	1.2	1.4	2.4
acidic		21.7	24.3	26.4	25.6	38.6
basic		14.9	9.8	11.2	9.2	6.4
diff		6.8	14.5	15.2	16.4	22.2

[a] Values expressed in mole percent.

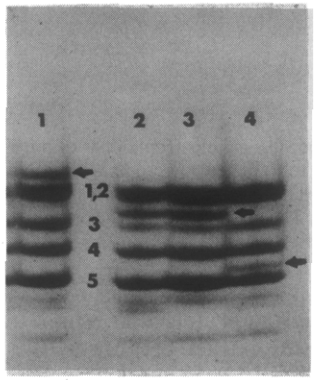

FIGURE 6: Electrophoresis of variant cuticle proteins in a gradient acrylamide gel: (1) slow-CP-1 variant, (2 and 3) CP-2/CP-3 variant, (4) slow CP-5 variant. Normal positions of CP-1 through CP-5 are indicated.

produced, CP-1 was absent from its standard position following electrophoresis in gradient gels. Genetic analysis indicates that the variant condition is specified by a gene carried on the second chromosome.

CP-2 and CP-3. A type of variant was found which resulted in the presence of an extra band at a position between CP-2 and CP-3 following electrophoresis on gradient gels. There was a reduction in the staining intensity of both CP-2 and CP-3. When homozygotes for the variant were produced, the CP-2 and CP-3 bands were both missing from their standard positions. It is not clear why only a single electrophoretic variant protein is found in the homozygous stock. It is possible that the variant condition results in an electrophoretic variant form of CP-2 or CP-3 which comigrates with one of the other major proteins (e.g., CP-1 or CP-4). However, no obvious differential increase in the staining intensity of the other bands has been detected. Densitometric tracings of gels with cuticle proteins from heterozygotes reveal that the variant band has greater staining intensity than either of the bands at the normal positions (data not shown, but see Figure 6), suggesting that the variant condition results in a single new electrophoretic form derived at the expense of both of the standard forms. For example, it is possible that CP-2, which has an apparent molecular weight slightly larger than CP-3, may be a precursor of CP-3. The variant condition may alter the electrophoretic mobility of CP-2 and prevent processing of CP-2 to form CP-3. Alternatively, CP-2 and CP-3 may share no common amino acid sequence but are derived from a precursor by proteolysis. This precursor may not be processed in the genetic variant and migrates between the normal CP-2 and CP-3 positions. The CP-2/CP-3 variant is specified by a gene carried on the second chromosome.

CP-5. A type of variant was found with an additional band between CP-4 and CP-5 after electrophoresis on gradient gels. Little apparent reduction in the intensity of staining of CP-4 or CP-5 was present in many of the heterozygotes in which the variant was identified (see Figure 6). When homozygotes were produced, and in some heterozygotes, a clear reduction in the staining intensity of CP-5 was noted, but even in homozygotes a band still occupied the CP-5 position. We conclude that the variant is a slow CP-5 and that there are two proteins (CP-5a and CP-5b) specified by at least two genes which comigrate during electrophoresis on gradient gels. Densitometric tracings of gels of proteins derived from homozygotes (data not shown) reveal that, at least for some lines, the variant electrophoretic form constitutes about 75% of CP-5a and CP-5b. Recent studies (Chihara and Fristrom, unpublished) have resulted in the detection of a variant of the second protein in a stock already containing a slow CP-5. Extensive genetic studies will be required to determine which of the two proteins, 5a or 5b, is altered in a particular variant isolate. For three independently isolated slow CP-5 variants, the variant condition is specified by a gene carried on the third chromosome.

The different variants were recovered at allele frequencies in the studied populations ranging from about 0.2% (CP-2/CP-3 variants) to 2% (CP-5 variants). These frequencies are near or below the frequency of allelic variations (1%) considered to be "polymorphic" by population geneticists (Harris and Hopkinson, 1972).

No variants of CP-4 were identified. No attempt was made to recover variants of any of the minor proteins.

Discussion

The urea-soluble proteins from unsclerotized third instar larval cuticles of *D. melanogaster* are similar to those of other insects. The solubility properties of these proteins, their affinity for chitin, and the high yield from larval cuticles following extraction with 7 MUT suggest that the proteins are of procuticle origin. A direct demonstration of the procuticle origin of these proteins using fluorescent antibody techniques has not yet been possible because attempts to produce antibody against the proteins by injecting rabbits with purified proteins in Freund's adjuvent have been unsuccessful. The 8000–10 000 mol wt fraction of the urea-soluble cuticle proteins is heterogeneous.

Insofar as the five major proteins are concerned, the evidence at hand indicates that three of the five are homogeneous (CP-1, 2, and 3), one may be heterogeneous (CP-5), and the fifth (CP-4) is probably homogeneous. The evidence for these conclusions comes from two sources. First, it has not been possible to demonstrate, in the four gel systems used, any subdivision of the major proteins. However, although the relative electrophoretic properties of some of the proteins differ in the presence and absence of urea (particularly CP-3 but also CP-4), the different systems are not markedly different in their separating properties. Also, since all of the proteins in the fraction have similar molecular weights, $NaDodSO_4$-gel electrophoresis probably would not resolve additional proteins. Thus, conclusions based on electrophoresis and chromatography are hardly sufficient. However, the genetic results pro-

vide a second line of evidence which suggests that several of the proteins are indeed homogeneous. Thus, the variants affecting CP-1, CP-2, and CP-3 when made homozygous all result in the elimination of these proteins from their normal positions. Accepting that these variants affect the primary structure of the polypeptides, then the results demonstrate that the three bands are homogeneous. It is unlikely that the variants involve enzymes which modify the polypeptides, since such mutants would be expected to be recessive and not recognizable in heterozygous form. The variant affecting CP-5 indicates that this band is composed of polypeptides derived from two different genes. It does not follow, however, that the two polypeptides are necessarily different proteins, although they may be different forms of the same protein. There are, for example, 13 genes for each of five histones in *Drosophila*. Hypothetically, there could be two genes, specifying "CP-5" proteins. Since no variants have been found affecting CP-4, there is no genetic evidence available regarding the homogeneity of this band. However, in view of the results with the other proteins and the shift of the CP-4 band in urea and native gels, it is likely that CP-4 is homogeneous. We have also identified in a laboratory stock a variant of CP-7 which eliminates this polypeptide from its normal position, indicating that it too is homogeneous.

Accepting the homogeneity of most of the proteins and in light of the amino acid compositions, we conclude that there are at least five polypeptides with different primary structures (CP-1, CP-2/CP-3, CP-4, CP-5, and CP-7) and perhaps as many as eight (also CP-6 and CP-8 and either CP-2 or CP-3) present in the urea-soluble protein fraction of cuticles. The existence of at least five polypeptides with different primary structures requires at least five different coding regions which specify these polypeptides. As used, the term "coding region" is not necessarily synonomous with "gene"; e.g., there are two coding regions for the two polypeptides in insulin, but potentially only one gene specifying the amino acid sequence of the insulin precursor. Thus, the possible existence of eight coding regions does not neccessitate eight genes, since at least two of the cuticle proteins (CP-2 and CP-3) appear to share a polypeptide region or be derived from a common precursor. Since the variants map to two different chromosomes, the evidence presented indicates that at least two genes are involved. Recent data (Chihara and Fristrom, unpublished) demonstrates that the CP-1 variants and the CP-2/CP-3 variants map at different positions on the second chromosome. Thus, in total at least three different genes are involved in the formation of the cuticle proteins.

We cannot agree with the proposal put forward by Hackman (1976) that the heterogeneity of the standard set of procuticle proteins is due to genetic polymorphism. In the case of *Drosophila,* the heterogeneity is a result of the existence of different polypeptides necessarily specified by different coding regions and not a result of the existence of multiple allelic forms of a single coding region. While our survey identified the existence of variant forms of cuticle proteins in natural populations, the level of polymorphism is low (less than 2%) and would not have been identified in crude extracts of cuticles derived from mass populations of larvae. Despite having properties similar to those of other insects, the *Drosophila* cuticle proteins are different, having different molecular weights and amino acid compositions. Thus, as already noted by Hackman (1976), procuticle proteins are not evolutionarily conserved throughout the Insecta. We have so far only examined one other species of *Drosophila, D. virilis,* a sibling species of *D. melanogaster,* and have found a set of procuticle proteins electrophoretically indistinguishable from that of *D. melanogaster.* Since the procuticle proteins are, by definition, structural proteins, they differ from other structural proteins, e.g., actin, histones, and collagen in undergoing rapid change during evolution. The lack of evolutionary conservation suggests that the role of the proteins in the structure of the procuticle is secondary to that of chitin.

The evidence presented in this paper indicates that at least three different structural genes, two located on the second chromosome and one located on the third chromosome, and perhaps as many as seven, are involved in specifying the amino acid sequences of the procuticle proteins. Since the formation of the procuticle is under the control of the steroid hormone ecdysone, which apparently acts via the genome, there is circumstantial evidence for the coordinate regulation of at least three physically separate procuticle protein structural genes. Since procuticle formation occurs during organ culture of epidermal tissues, including mass-isolated imaginal disks of *D. melanogaster* (Fristrom, unpublished), the opportunity exists for studying the coordinate synthesis of procuticle proteins in vitro. Except for CP-2/CP-3, we have no evidence yet to indicate that common precursors exist for different urea-soluble procuticle proteins. Since the deposition of the procuticle involves secretory activity by the underlying epidermal cells (Locke, 1976; Mitchell et al., 1971), we anticipate the existence of precursors of the procuticle proteins which are soluble in nondissociating solvents.

Acknowledgments

We are grateful to D. MacPheason and D. Silvert for technical assistance, to D. Fristrom for providing the micrographs, H. Hoffman for the use of his facilities, and to M. A. Yund for helpful comments during the preparation of the manuscript. J. W. F. is particularly indebted to Drs. I. Franklin and J. Sved for an introduction to the techniques and pleasures of sampling wild populations of *D. melanogaster* in the wineries of the Hunter Valley.

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Chemical Modification Studies on the Ca^{2+}-Dependent Protein Modulator: The Role of Methionine Residues in the Activation of Cyclic Nucleotide Phosphodiesterase†

Michael Walsh and Frits C. Stevens*

Appendix: Circular Dichroism Studies on Ca^{2+}-Dependent Protein Modulator Oxidized with *N*-Chlorosuccinimide

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ABSTRACT: Methionine residues have been implicated in the activation of cyclic nucleotide phosphodiesterase by the Ca^{2+}-dependent protein modulator [Walsh, M., & Stevens, F. C. (1977) *Biochemistry 16,* 2742–2749]. Treatment of the modulator with *N*-chlorosuccinimide in the presence of Ca^{2+} resulted in selective oxidation of methionine residues at positions 71, 72, 76, and, possibly, 109 in the modulator sequence. These residues lie on the surface of the molecule exposed to solvent. This modification has several effects on the modulator protein: (1) the Ca^{2+}-binding properties of the oxidized modulator are changed with apparent loss of high-affinity binding sites, (2) the oxidized protein no longer interacts with phosphodiesterase, and (3) troponin C like activities, viz., Ca^{2+}-dependent change in mobility on urea–polyacrylamide gel electrophoresis and formation of a urea-stable complex with troponin I, are lost upon oxidation of the modulator. The phosphodiesterase binding domain of the modulator protein appears to be located between the second and third Ca^{2+}-binding loops, a region of the molecule known from previous partial proteolysis studies [Walsh, M., Stevens, F. C., Kuznicki, J., & Drabikowski, W. (1977), *J. Biol. Chem. 252,* 7440–7443] to be exposed in the presence of Ca^{2+}.

Considerable evidence has been provided in recent years that a ubiquitous Ca^{2+}-binding protein, originally discovered as an activator of a cyclic nucleotide phosphodiesterase by Cheung (1970, 1971) and Kakiuchi et al. (1970) in rat brain and by Goren & Rosen (1971) in bovine heart, may serve as the central regulator which couples Ca^{2+} to the regulation of cyclic nucleotide metabolism and nonmuscle contractile and secretory processes. This so-called modulator protein exhibits many Ca^{2+}-dependent regulatory activities in addition to its stimulation of phosphodiesterase: (1) activation of a specific adenylate cyclase (Brostrom et al., 1975; Cheung et al., 1975); (2) troponin C like activity with a reconstituted Ca^{2+}-sensitive actomyosin ATPase system (Amphlett et al., 1976; Dedman et al., 1977); (3) activation of smooth-muscle myosin light-chain kinase (Dabrowska et al., 1978), skeletal-muscle protein kinase (Yagi, et al., 1978), and brain protein kinase (Schulman & Greengard, 1978); (4) activation of erythrocyte membrane (Ca^{2+},Mg^{2+})ATPase (Jarrett & Penniston, 1977; Gopinath & Vincenzi, 1977); and (5) stimulation of erythrocyte-membrane Ca^{2+} transport (Macintyre & Green, 1977). Furthermore, an unidentified protein, which appears to be an additional modulator-regulated enzyme, has been isolated from brain and shown to interact with the modulator in a Ca^{2+}-dependent manner (Wang & Desai, 1976, 1977). These modulator-regulated enzymes may contain a common modulator-binding domain.

The modulator protein exhibits none of the above-mentioned activities in the absence of Ca^{2+} ions. The protein binds four Ca^{2+} ions per mole, and the binding of Ca^{2+} is accompanied

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0006-2960/78/0417-3924$01.00/0