Pupal and Larval Cuticle Proteins of *Drosophila melanogaster*†

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ABSTRACT: Proteins, soluble in 7 M urea, were extracted from third-instar larval and pupal cuticles of *Drosophila melanogaster*. Both extracts contain a limited number of polypeptides resolved by one- or two-dimensional electrophoresis. The five major larval proteins have low molecular weights (<20 000) and are not glycosylated. The major pupal cuticle proteins fall into two size classes: two with apparent molecular weights of 56K and 82K and four with molecular weights between 15K and 25K. The proteins with high apparent molecular weights are glycosylated. In nondenaturing gels, no components of the larval and pupal cuticle extracts comigrate. One-dimensional "fingerprints" indicate that cuticle proteins from these two stages have unique primary structures. Immunological results indicate that the major low molecular weight larval and pupal cuticle proteins are comprised of two families of proteins that share antigenic determinants. The high molecular weight pupal cuticle proteins are immunologically unrelated to the low molecular weight components. We conclude that the pupal and larval proteins are encoded in part by multigene families that have arisen by gene duplication and evolutionary divergence.

The cuticle of *Drosophila melanogaster* is an extracellular structure that is deposited anew prior to each of four postembryonic molts by an underlying epithelium. Each molt is triggered by the steroid 20-hydroxyecdysone (Borst et al., 1974; Hodgetts et al., 1977; Berreur et al., 1980). The third-instar larval cuticle is a product of the larval epithelium. This cuticle is not shed at the larval–pupal molt; rather, concomitant with spiracle eversion, the cuticle contracts to form a barrellike structure and subsequently becomes tanned (Fraenkel, 1935; Fraenkel & Ruddall, 1947). The restructured third-instar larval cuticle is called the puparium, and the process by which it is formed is termed pupariation (Fraenkel & Bhaskaran, 1973). Following the formation of the puparium, a pupal cuticle is deposited by the imaginal disks in the head and most of the thorax, and by the larval epithelium and abdominal histoblasts in the abdomen of the pharate pupa (Whitten, 1968; Roseland & Schneiderman, 1979; Madhavan & Madhavan, 1980). Thus, the pupal cuticle is the product of a mosaic epithelium.

The general structure of the cuticle is the same throughout the animal's development (Poodry, 1980). The outer layer, the epicuticle, is secreted first and consists of an electron-dense layer, called the cuticulin, and a broader, less electron-dense layer, called the inner epicuticle (Locke, 1974). Neither region of the epicuticle is well described biochemically. However, the epicuticle is believed to consist largely of quinone-stabilized proteins and waxes (Poodry, 1980). The inner layer, the procuticle, is a lamellate structure that consists of the nitrogenous polysaccharide chitin and proteins in roughly equal proportions.

We have been investigating the genetics, synthesis, and properties of a subset of cuticle proteins insoluble in neutral buffers of moderate ionic strength but soluble in denaturing agents such as 7 M urea or 1% sodium dodecyl sulfate (SDS).[1] The urea-soluble third-instar larval cuticle proteins of *Drosophila*, first described by Fristrom et al. (1978), are encoded by at least five genes that are active in the epidermis of third-instar larvae (Fristrom et al., 1978; Snyder et al., 1981). The physical role these proteins play in the cuticle is unknown although the renatured proteins bind to chitin at pH 7 (Fristrom et al., 1978). A previous report on the developmental profile of cuticle proteins in *Drosophila* (Chihara, et al., 1982) describes qualitative differences in the urea-soluble fractions of all stages. In this report, urea-soluble proteins derived from mass-isolated third-instar larval and pupal cuticles are compared by utilizing biochemical and immunological techniques.

Experimental Procedures

A wild-type laboratory strain of *Drosophila melanogaster* (Oregon R) that has been maintained in mass culture in the Genetics Department at Berkeley since 1964 was used in these studies. Gram quantities of third-instar larvae and pupae were raised and recovered according to Mitchell & Mitchell (1964). All *Drosophila* cultures were maintained at 25 °C.

Preparation of Cuticles. Larval cuticles were prepared according to Fristrom et al. (1978). Pupal cuticles were prepared similarly. Liter quantities of pupae were collected 10–22 h after puparium formation and were either frozen at −70 °C or extracted immediately, as follows: Pupae were homogenized in Ringer's solution that contained 1 mM PMSF. After a second extraction with a high-salt buffer (0.5 M NaCl and 5 mM Tris–maleate, pH 7.0), the insoluble material consisted of cleaned puparial and pupal cuticles that are easily distinguished from one another because only the puparial cuticles are tanned. The pupal and puparial cuticles were then separated by virtue of their differing buoyant densities [suggested by Fukushi & Seki (1965), who report that the "pupal sheath is collected by a floating method"]. The sclerotized puparial cuticles sink in 8.5% NaCl whereas the unsclerotized pupal cuticles float. The floating cuticles were placed in 8.5% NaCl a second time to displace any puparial cuticles trapped with the pupal cuticles. A final immersion of the floating

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[1] Abbreviations: DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; Tris–urea buffer, 7 M urea–0.005 M Tris. pH 8.6; phosphate-buffered saline, 0.01 M phosphate–0.15 M NaCl, pH 7.2; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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cuticles resulted in a preparation that was greater than 95% transparent pupal cuticle. The resulting cuticle preparation was free of contaminating tissues when viewed at 50× with a dissecting microscope.

Protein Extraction. Cuticle preparations were extracted with Tris–urea buffer as reported by Fristrom et al. (1978). Alternatively, cuticles were extracted in 1% SDS and then precipitated with 4 volumes of acetone. Following extensive washing with acetone, the precipitated proteins were dried under vacuum and then redissolved in Tris–urea buffer or SDS sample buffer (Laemmli, 1970).

Protein Determination. Two methods were routinely used. In the first, total protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. In the second method, total protein was determined by the absorbance of protein solutions at 280 nm. An extinction coefficient of 1 absorbance unit = 0.55 ± 0.04 mg of protein/mL was determined empirically for the third-instar larval cuticle proteins and assumed operative for the pupal cuticle proteins.

Carbohydrate Determination. The quantity of neutral sugars attached to cuticle proteins was estimated by using the phenol–sulfuric acid method of Dubois et al. (1956). Glucose and mannose were used to generate standard curves; ovalbumin and lysozyme were used for positive and negative controls, respectively. The nature of monosaccharides present in pupal cuticle proteins was determined by hydrolysis in 4 M trifluoroacetic acid for 3 h at 105 °C (Lundblad et al., 1975) using mannan as a control. The hydrolysates were chromatographed on an anion-exchange column (AG11 A-8). Column fractions were lyophilized, and the monosaccharides were separated by paper or thin-layer chromatography together with standards. The solvent was 1-butanol–pyridine–water (10:3:3). Reducing sugars were identified with alkaline $AgNO_3$.

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

(*A*) *Nondenaturing.* We use the term nondenaturing electrophoresis to describe the electrophoresis of urea-denatured proteins in an otherwise native gel system. Nondenaturing gel electrophoresis was performed in slab polyacrylamide gels (1.5 mm × 15 cm × 22 cm). The separation gel of this system is prepared in a solution of 75 mM Tris-HCl, pH 8.6, and consists of 14.6% acrylamide and 0.4% bis(acrylamide). Samples were introduced for electrophoresis in Tris–urea buffer. The outside wells were filled with 5 μL of a 0.1% solution of bromphenol blue in Tris-urea buffer as markers for electrophoretic migration. The electrode buffer was either Tris–glycine (0.025 M Tris–0.67 M glycine) or 0.3 M borate, both at pH 8.5. Electrophoresis was performed at room temperature at a constant voltage of 100 V for the first 30 min and then at 150 V until the dye front had migrated a minimum of 13 cm. The gel was then stained for protein as described below.

(*B*) *Denaturing.* Denaturing gel electrophoresis using sodium dodecyl sulfate (SDS) as the denaturant was performed according to the method of Anderson et al. (1973) in 14.6% acrylamide and 0.4% bis(acrylamide) gels using molecular weight standards as indicated in the figure legends.

(*C*) *Two Dimensional.* For the second dimension, the lane of a nondenaturing gel was cut out and used immediately or placed in plastic wrap, frozen, and used at a later time. The strip was placed with 2–2.5 volumes of SDS sample buffer (Laemmli, 1970) and shaken at room temperature for 1.5 h. The strip was then placed at the top of a denaturing gel, and electrophoresis was conducted as already described. Proteins were stained with Coomassie Blue following the method of Fairbanks et al. (1971).

One-Dimensional Peptide Map. Cuticle proteins identified in nondenaturing gels were subjected to peptide mapping by limited proteolysis in denaturing gels (Cleveland et al., 1977). Individual bands were cut from the nondenaturing gel, soaked in sample buffer (0.5% SDS, 10% glycerol, 0.0001% bromphenol blue, and 0.125 M Tris-HCl, pH 6.8), and frozen at −70 °C. The prepared gel slice was then placed in a slot of a denaturing (SDS) 20% polyacrylamide gel with a 5% stacking gel. Layered atop the slice was 100 ng of V.8 protease (Miles Laboratories) dissolved in 5 μL of sample buffer. Electrophoresis was commenced at 100 V until the dye front just entered the stacking gel, at which time the power supply was switched off. The gel was then placed in an incubator at 37 °C for 1 h. Electrophoresis was then resumed.

Chromatography. Column chromatography was carried out as follows.

(*A*) *Ion-Exchange Chromatography.* DEAE-cellulose (Whatman DE-52) chromatography was used to partially purify and concentrate crude cuticle protein preparations. Procedures specified in Fristrom et al. (1978) were followed for preparing and loading columns. Proteins were displaced from DEAE with 0.1 M NaCl in Tris–urea buffer.

(*B*) *Gel Filtration.* Gel filtration was performed by using a Sephacryl S-200 (Pharmacia) column (2.5 × 45 cm) that was preequilibrated with phosphate-buffered saline. With a pressure head of 60 cm, 2-mL fractions were collected every 2.5 min. The column was calibrated with known molecular weight markers dissolved in Tris–urea buffer.

(*C*) *Binding to Chitin.* Columns of purified chitin were used following the procedure of Fristrom et al. (1978). We also investigated whether successive washes of increasing pH eluted cuticle proteins. To that end, different buffers (5 mM Tris-HCl and 5 mM NaCl) adjusted between pH 7 and pH 9 in 0.5-unit increments were prepared and used.

Immunological Methods. (*A*) *Antibody Production and Preparation.* Complex antisera were prepared against both larval and pupal cuticle proteins. Because initial experiments indicated that cuticle proteins are poor immunogens (Downe, 1962; Fristrom et al., 1978), they were cross-linked by glutaraldehyde to increase their immunogenicity (Reichlin et al., 1970). Two types of antisera were prepared against the pupal cuticle proteins. In the first, the pupal cuticle proteins were fractionated on a molecular sieve (see above and Results) to remove the high molecular weight components rich in carbohydrates that may have dominated the immune response in the rabbits. In the second, unfractionated, un-cross-linked pupal cuticle proteins were used as immunogens. Third-instar larval cuticle proteins were prepared by displacement with 0.1 M NaCl from DEAE-cellulose. Emulsions were made with complete Freund's adjuvant (supplemented with additional *Mycobacterium*, 4 μg/mL) in the ratio of 1 mL of protein solution to 1 mL of adjuvant. Initial injections were made intradermally in three sites of the rabbit—the back and each hindquarter. Booster injections of antigen dissolved in phosphate-buffered saline were made intravenously in the marginal ear vein. For the production of the second antipupal cuticle protein serum, booster injections in incomplete Freund's adjuvant were made subcutaneously at three sites on the back of the animal. Rabbits were bled 8–12 days after each booster injection. The serum was recovered and clarified according to standard procedures described by Campbell et al. (1963). When necessary, antibodies were partially purified and concentrated from sera by ammonium sulfate precipitation

(Campbell et al., 1963). Otherwise, sera were stored whole in 1-mL aliquots at −20 °C.

(*B*) *Immunoelectrophoresis.* Immunoelectrophoresis was performed in Tris-HCl buffer (0.1 M, pH 8.6) at 1–2 V/cm for at least 6 h. An appropriate amount of antiserum was placed into a 1% solution of agarose at approximately 55 °C and then poured onto a microscope slide (details are provided in the figure legends). Antigens migrated during electrophoresis into an antibody-containing gel from a strip of acrylamide gel containing proteins previously separated by either nondenaturing or denaturing (SDS) gel electrophoresis (crossed immunoelectrophoresis). Strips of denaturing (SDS) gels were equilibrated for 4 h in about 100 volumes of 4 M urea, 100 mM Tris, and 1% Triton X-100 (v/v), pH 8.6, and then for 4 h in the same solution without urea. This treatment was required to eliminate artifacts resulting from SDS. For line electrophoresis, antigens migrated from a strip of filter paper (Whatman 1, 0.125 in. wide). Alternatively, antigens were added to melted agarose that was quickly poured on a glass plate or microscope slide. Electrophoresis into antibody-containing agarose was then performed. Where the equivalence point is reached between antibody and antigen concentrations, a precipitin line forms, thereby resulting in characteristic waves, lines, and deflections from lines. A standard reference for these techniques is Axelsen et al. (1973).

Results

Yields of Urea-Soluble Cuticle Proteins. Gram quantities of third-instar larval and pupal cuticles were recovered. Cuticle proteins not extracted by neutral buffers of low or moderate ionic strengths, but extracted by denaturants, were recovered in Tris–urea buffer. Prepared larval cuticles yield 21.6 ± 1.8% of their dry weight in the Tris–urea buffer soluble fraction whereas pupal cuticles yield only 4.9 ± 0.2%. Determinations were made by measuring the mass of the dried cuticle before and after extraction and by measuring the amount of protein in the extraction fluid. The apparent difference in the amount of cuticle proteins per unit cuticle between larval and pupal cuticles deserves comment. By electron microscopy, the epicuticles of both stages appear the same, but the pupal procuticle is far smaller than the procuticle of the third-instar larva. Thus, the lower yield of urea-soluble pupal cuticle proteins per dry weight may result in part from a lesser contribution of the procuticle to the total weight of the pupal cuticle than to the larval cuticle.

Electrophoretic Characterization of Major Cuticle Proteins. The results of electrophoresis of urea-soluble larval and pupal cuticle proteins are displayed in Figure 1. Proteins are numbered as a function of their migration in nondenaturing gels (A and B) and, in the case of pupal cuticle proteins, as a function of their apparent molecular weights in denaturing gels (C). The third-instar larval cuticle proteins have previously been shown to have apparent molecular weights between 9K and 20K (Fristrom et al., 1978). A distinction between the pupal cuticle proteins and the third-instar larval cuticle proteins is that the six major pupal cuticle proteins resolve into two classes under denaturing conditions. Four major pupal cuticle proteins have apparent molecular weights between 15K and 25K; two others have apparent molecular weights of 56K and 82K. The latter two proteins are glycosylated (see below). Another distinction between third-instar larval cuticle proteins and pupal cuticle proteins is their differential behavior when subjected to nondenaturing electrophoresis in the presence of borate. Borate has long been used to identify sugars that have vicinal hydroxyls (Boeseken, 1949). Two recent reports indicate that borate improves resolution of electrophoretic

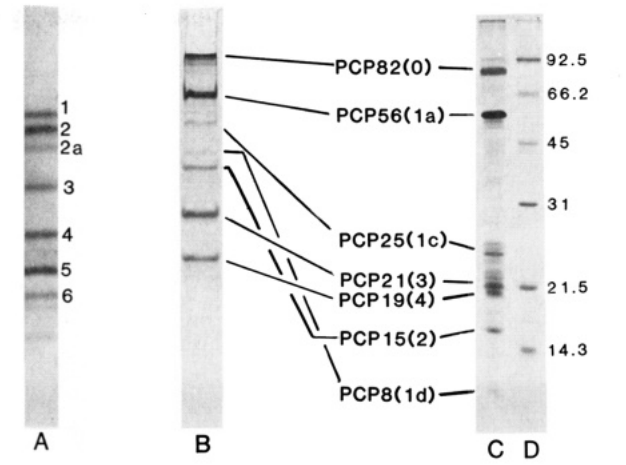

FIGURE 1: One-dimensional electrophoresis of cuticle proteins. Lanes A and B show a nondenaturing polyacrylamide gel of larval cuticle proteins (lane A) and pupal cuticle proteins (lane B). Electrophoresis was in Tris–glycine (pH 8.6) running buffer; samples were introduced in Tris–urea buffer. Lane C shows a denaturing polyacrylamide gel of the pupal cuticle proteins. The nomenclature of the pupal cuticle proteins reflects their apparent molecular weights on the basis of standards (lane D). The previously used nomenclature for pupal cuticle proteins (Chihara et al., 1981) is shown in parentheses. The relationships between pupal cuticle proteins separated by nondenaturing and denaturing gels were determined by excising pupal cuticle protein bands from a nondenaturing gel and subjecting them to electrophoresis under denaturing conditions (also see Figure 2). Proteins were stained with Coomassie Brilliant Blue.

analysis of glycopeptides (Weitzman et al., 1979; Narasimhan et al., 1980). A two-dimensional analysis of the pupal cuticle protein borate pattern (Figure 2C) reveals that many of the high molecular weight pupal cuticle proteins [e.g., pupal cuticle protein 1a (56)] are retarded in their electrophoretic migration in borate. In addition, protein 1d (8) is retarded, and protein 2 (15) is resolved into two species. We shall discuss these results further when we consider the glycosylation of cuticle proteins. In contrast to pupal cuticle proteins, the third-instar larval cuticle proteins are unaltered in their relative electrophoretic migration in borate (data not shown).

Partial Purification of Pupal Cuticle Proteins. Proteins in the crude urea extract of pupal cuticles were concentrated by displacement from DEAE-cellulose with 0.1 M NaCl. Samples were then subjected to gel filtration using a Sephacryl S-200 column equilibrated with phosphate-buffered saline. As pictured in Figure 3, the crude preparation was fractionated into three classes. Peak I material eluted at the void volume and contains those proteins that barely enter the nondenaturing polyacrylamide gel (protein O). Peak II material contains most of the major pupal cuticle proteins, i.e., pupal cuticle proteins 15, 19, 21, 25, and 56. Peak III material is enriched (curiously) for protein 56.

Glycosylation of Cuticle Proteins. The results from two-dimensional electrophoresis in the presence of borate (Figure 2C) suggest that pupal cuticle proteins 82, 56, 15, and 8 may be glycoproteins. The carbohydrate content of the third-instar larval cuticle proteins and pupal cuticle proteins was investigated by using the phenol–sulfuric acid assay. The results, tabulated in Table I, indicate that the third-instar larval cuticle proteins have a low level (about 0.2%) of glycosylation whereas the pupal cuticle proteins have a substantially higher level (about 1.4%). When purified fractions of third-instar larval cuticle proteins (Fristrom et al., 1978) are used, only third-instar larval cuticle protein 3 has detectable sugar by this method—about 0.2%. This level of glycosylation is equivalent to 1 molecule of glucose per 10 protein molecules and hence

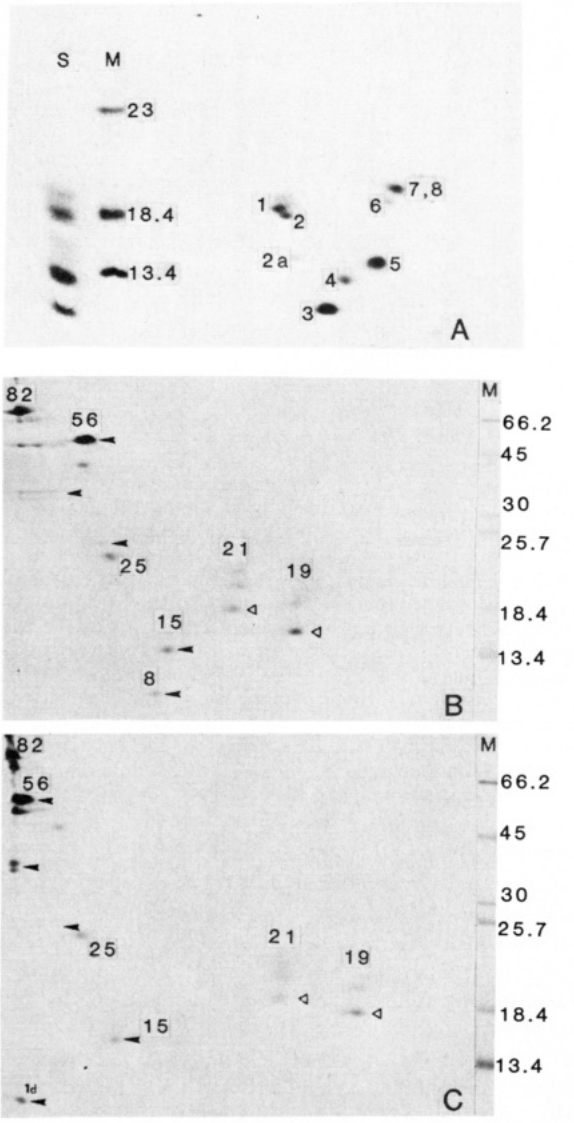

FIGURE 2: Two-dimensional electrophoresis of larval cuticle proteins (A) and pupal cuticle proteins (B and C). The first dimension, migration from left to right, used nondenaturing conditions. The second dimension, migration from top to bottom, used denaturing conditions. (A) Lane S contains larval cuticle proteins separated by denaturing conditions in one dimension. (A–C) Lane M contains molecular weight markers. Proteins are numbered as in Figure 1. Where multiple bands are present, the major one, named in Figure 1, is identified (open arrow). The Tris–glycine buffer was used for the first dimension. Gel C duplicates gel B, but borate is present in the running buffer. Peptides (closed arrows) whose migration is retarded in the presence of borate include components of pupal cuticle proteins 8, 15, and 56 and two minor peptides.

Table I: Carbohydrate Contents of Cuticle Proteins[a]

LCP	carbohydrate	PCP	carbohydrate	monosaccharides
total	0.19	total	1.4	HxNAc, Glc, Gal, Man
1, 2, 4, 5	0.0	82	7.4	GlcNAc, GalNAc, Glc, Gal, Man
3	0.18	56	2.8	
		15, 19, 21, 25	0.6	

[a] Carbohydrate content is given in milligrams of carbohydrate per 100 mg of protein. Abbreviations: LCP, larval cuticle protein; PCP, pupal cuticle protein; HxNAc, *N*-acetylhexosamine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Glc, glucose; Gal, galactose; Man, mannose. LCP fractions were highly purified (Snyder et al., 1982). PCP fractions were enriched by gel filtration.

may result from contamination. Indeed, direct sequencing by Snyder et al. (1982) detects no sugar moieties attached to the

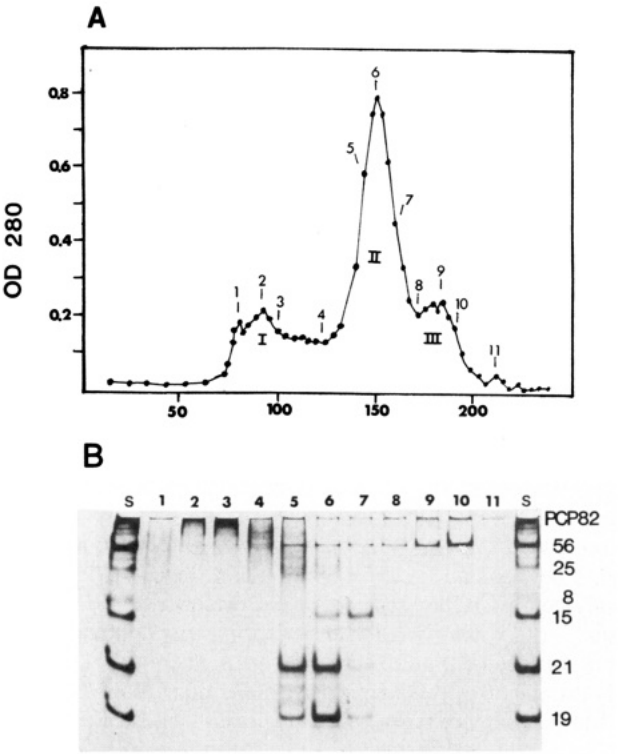

FIGURE 3: Gel filtration of pupal cuticle proteins. Pupal cuticle proteins, concentrated by displacement from DEAE-cellulose by 0.1 M NaCl in urea–Tris buffer, were loaded directly onto a Sephacryl S-200 column (2.5 × 45 cm) equilibrated with phosphate-buffered saline. Two-milliliter fractions were collected. (A) Elution profile from S-200. Numbers refer to samples analyzed by electrophoresis. (B) Nondenaturing electrophoresis of samples of eluate. S, unfractionated pupal cuticle proteins. Numbers refer to fractions in (A).

first 72 of the 96 residues of this protein. However, an asparagine at residue 92 is a possible site for N-linked glycosylation (Asn-X-Ser). When pupal cuticle proteins partially purified by gel filtration are used, the majority of the detectable glycosylation resides with the proteins with high apparent molecular weights (protein 82, 7.6% glycosylation; protein 56, 2.8% glycosylation). The fraction containing the low molecular weight pupal cuticle proteins 15, 19, 21 and 25 was found to have 0.6% glycosylation. Because of the presence of contaminating protein 56, we assume that these low molecular weight species contain little or no sugar. In this respect, the low molecular weight pupal cuticle proteins emulate the third-instar larval cuticle proteins which also contain little or no carbohydrate. Despite their altered electrophoretic migration in borate, we find no corroborating evidence indicating proteins 8 and 15 are glycosylated.

After acid hydrolysis, the monosaccharides liberated from pupal cuticle proteins were separated on an ion-exchange column and then subjected to thin-layer and paper chromatography together with standards. The main components identified on the basis of comparable migration are listed in Table I. Hexosamines and mannose were found in higher amounts than galactose and glucose. The possible presence of glycogen-like molecules associated with the pupal cuticle proteins was eliminated by the failure of glycogen phosphorylase to liberate detectable glucose 1-phosphate under a variety of incubation conditions (data not shown). Traces of hexuronic acid like, mannosamine like, and fucose like substances were also detected in the hydrolysates.

One-Dimensional Peptide Maps. One-dimensional peptide maps of the major third-instar larval cuticle proteins and pupal cuticle proteins as delineated in nondenaturing gels are

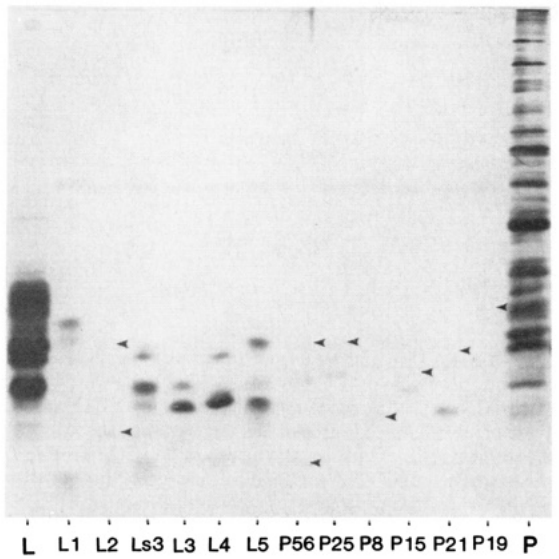

FIGURE 4: One-dimensional peptide maps of major larval cuticle protein and pupal cuticle protein polypeptides (see Experimental Procedures for details). Lane L contains undigested larval cuticle proteins; lane P is undigested pupal cuticle proteins. Larval (L) and pupal (P) peptides are indicated. Ls3 is a variant peptide. Some peptide fragments with indistinguishable mobilities from those in other lanes are apparent. Fragments visible in the gel, but not readily seen in the photograph, are identified by arrows.

presented in Figure 4. All peptide profiles are distinctive. However, there are some peptide fragments with indistinguishable mobilities. In particular, fragments that migrated about 8 cm (R_f = 0.67) are found in digests of larval protein 3, larval protein 5, pupal protein 15, pupal protein 25, and pupal protein 56. Fragments that migrated about 8.5 cm (R_f = 0.71) are common to larval proteins 3, 4, and 5. Although digests of larval protein 3 and larval protein 5 contain fragments that migrate equivalent distances, the two larval proteins are, on the basis of immunological studies (see below), unlikely to have the same primary structures. Overall, the peptide maps suggest that many of the third-instar larval cuticle proteins and pupal cuticle proteins have distinct primary structures and are encoded by discrete genes.

Binding to Chitin. Figure 5 displays results from chitin binding experiments with the third-instar larval cuticle proteins and pupal cuticle proteins. In both cases, some material did not adsorb to the chitin and eluted with the pH 7.0 buffer. The unbound material amounted to 13.3% for the third-instar larval cuticle proteins and 30.4% for the pupal cuticle proteins. When concentrated and subjected to electrophoresis in a nondenaturing gel, the larval flow-through migrated with minor components, some hitherto unseen. The pupal flow-through consisted of proteins 8, 56, and 82, and to a much lesser extent proteins 19 and 21. Little protein was dislodged by increasing the pH of the elution buffers up to 9.0. The pH 9.0 buffer displaced 8–9% of the total loaded protein and included many, if not all, of the same minor components that eluted in the previous washes. The final solution applied to the column, Tris-urea buffer, released nearly all the remaining proteins (35–38%), resulting in a recovery of 98–99%. For the experiment involving third-instar larval cuticle proteins, all major proteins and some minor ones were included in the Tris–urea buffer fraction. The pupal cuticle proteins eluted by Tris–urea buffer included the major low molecular weight proteins (pupal cuticle proteins 15, 19, 21, and 25) and many minor ones.

Immunoelectrophoresis. Two types of studies were done. In one, the relationships between the low molecular weight

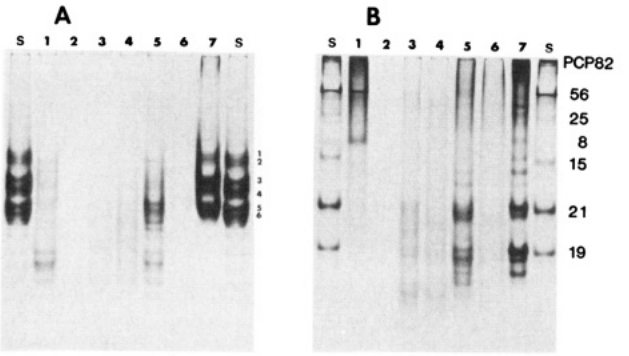

FIGURE 5: Chitin affinity chromatography of larval cuticle proteins and pupal cuticle proteins. Proteins were exhaustively dialyzed against binding buffer (5 mM NaCl–5 mM Tris-HCl, pH 7.0) and then loaded onto a column (1 × 2 cm) containing chitin. After 3 h at room temperature, the column was washed with 12-mL aliquots of the following: (1) binding buffer at pH 7.0, (2) 7.5, (3) 8.0, (4) 8.5, and (5) 9.0; (6) binding buffer with 0.5 M NaCl; (7) Tris–urea buffer. Samples from each wash were acid precipitated and subjected to electrophoresis under nondenaturing conditions. Lanes labeled S contain (A) larval cuticle proteins and (B) pupal cuticle proteins.

third-instar larval cuticle proteins and pupal cuticle proteins were investigated. In the other, the relationships between the low molecular weight cuticle proteins and the high molecular weight pupal cuticle proteins were investigated.

(A) Relationships among the Low Molecular Weight Cuticle Proteins. Crossed immunoelectrophoresis was performed to determine which of the pupal and larval cuticle proteins were recognized by the two antisera. The proteins were first separated in one dimension in nondenaturing gels. The separated proteins were then subjected to electrophoresis into an antibody-containing gel where precipitin bands formed. As seen in Figure 6A, all major third-instar larval cuticle proteins are recognized by antibody made against the larval proteins. However, in Figure 6B, only pupal cuticle proteins 15, 19, 21 and 25 are clearly recognized by the anti-pupal cuticle protein serum used here. We presume this results from the removal of the high molecular weight material from the crude pupal cuticle protein preparation used to raise this antiserum. The one-dimensional gels pictured at the bottom of Figure 6A,B are only representative of the ones from which the proteins migrated into the antibody-containing gel. Further information can be gained from Figure 6A. Because the "rockets" of proteins 1 and 2 have a fused intersection at their bases, as do larval proteins 3 and 4, it is evident that these two pairs of proteins share antigenic determinants. Whether the bases of the rockets from larval protein 2 and larval protein 3 fuse is not clear here. In contrast, larval protein 5 shows nonidentity with larval protein 4 (hence with larval protein 3 as well) in that the precipitin lines of the two proteins cross. The results in Figure 6C determine more precisely the relationship between individual larval proteins and the low molecular weight pupal cuticle proteins. Here, third-instar larval cuticle proteins, separated in a nondenaturing gel, migrated with a "line" of pupal cuticle proteins into a gel containing antibody against the larval cuticle proteins. The pupal cuticle proteins form two distinct precipitin lines. Larval proteins 1, 2, 3, and 4 partially deflect the lower line. Thus, these four proteins share partial immunological identity and are related to some of the pupal cuticle proteins. The peak formed by larval protein 5 deflects the second line, indicating antigenic relatedness to a pupal cuticle protein. Larval protein 2a comprises yet a third antigenic group because it forms a rocket (arrow) that does not deflect either of the pupal cuticle protein precipitin lines. Thus, only larval protein 2a is unique immunologically to the

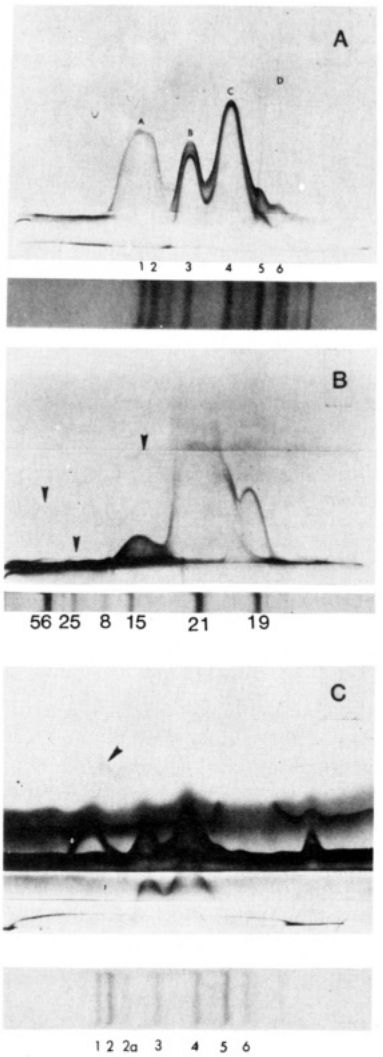

FIGURE 6: Immunoelectrophoresis of cuticle proteins. Proteins were first separated by nondenaturing electrophoresis. (A) Larval cuticle proteins were separated by nondenaturing electrophoresis in the first dimension. The second-dimension gel contained 75 μL/mL anti-larval cuticle protein. Peak A contains larval cuticle proteins 1 and 2; peak B, larval cuticle protein 3; peak C, larval cuticle protein 4; peak D, larval cuticle proteins 5 and 6. (B) Pupal cuticle proteins were separated by nondenaturing electrophoresis in the first dimension. The second-dimension gel contained 75 μL/mL anti-pupal cuticle protein (at the bottom) and 125 μL/mL at the top. Faint peaks identified by arrows appear above pupal cuticle protein 56, 25, and 15. Peaks above proteins 15, 19, and 21 are readily visible. (C) cross-line immunoelectrophoresis. Larval cuticle proteins were separated by electrophoresis as in (A). A line of pupal cuticle proteins was introduced from a narrow strip of Whatman 1 filter paper that contained about 2 ng of pupal cuticle proteins. The second-dimension gel contained 100 μl/mL anti-larval cuticle protein sera. Precipitin peaks associated with larval cuticle proteins 1, 2, 3, and 4 all deflect the same line. Larval cuticle protein 5 deflects another line. Larval cuticle protein 2a is associated with a precipitin peak (arrow) that deflects no major pupal protein line. The one-dimensional gels shown in panels A–C are representative of the gels used to produce precipitin bands but are not perfect matches.

larval set; all others share some immunological relatedness with various pupal cuticle proteins. Also, the two major sets of larval antigenic determinants (in larval proteins 1–4 and larval protein 5) are present in the pupal cuticle proteins.

(B) Relationships between Low and High Molecular Weight Cuticle Proteins. The immunological relatedness of larval and pupal cuticle proteins is examined further in Figure 7. Here the antiserum was raised against the entire set of pupal cuticle proteins. Two major pupal cuticle protein precipitin lines form

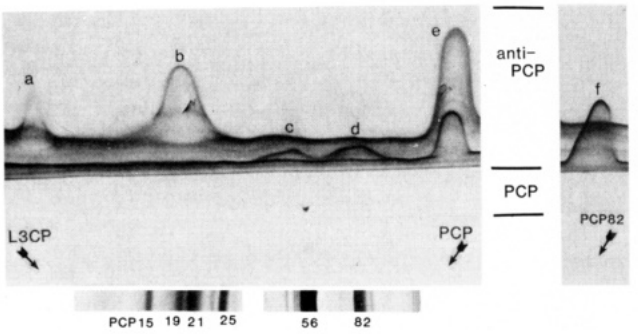

FIGURE 7: Crossed-line and rocket-line immunoelectrophoresis of pupal cuticle proteins. Pupal cuticle proteins (1.3 μg/mL) were introduced from strips (labeled PCP) of 1% agarose containing 0.5% Triton X-100 and were electrophoresed into a 1% agarose gel (labeled anti-PCP) that contained 5.3% of each of the anti-pupal cuticle protein rabbit antisera described under Experimental Procedures. In the left panel, pupal cuticle proteins were first separated by denaturing (SDS) gele electrophoresis. The region of the gel containing pupal cuticle proteins 56 and 82 was cut from a gel loaded with 100 μg of total pupal cuticle proteins. The region containing the low molecular weight pupal cuticle proteins was cut from a gel lane loaded with 2 μg of total pupal cuticle proteins. The one-dimensional gels shown at the bottom of the figure are representative of the gels used to produce the precipitin bands. Rocket b contains at least pupal cuticle proteins 19 and 21. Rocket d contains protein 82. Rocket c is caused by proteins migrating faster than protein 56; protein 56 does not migrate into the antibody-containing gel under the conditions used. Rocket-line immunoelectrophoresis utilized antigens migrating from wells punched into the agarose (arrows). Larval cuticle proteins (0.1 μg) formed rocket a and deflected only the top line. Pupal cuticle proteins (2 μg) formed rocket e and deflected both lines. Partially purified protein 82 (1 μg) formed rocket f. (Protein 82 was partially purified by size-exclusion chromatography and contains contaminants of other high molecular weight pupal cuticle proteins, but no low molecular weight proteins.) Only the lower precipitin line is deflected.

upon electrophoresis of pupal cuticle proteins into a gel containing anti-pupal cuticle protein serum. The low molecular weight pupal cuticle proteins, originating from a denaturing gel, deflect only the upper precipitin line. The lower precipitin line, however is deflected by protein 82 and other high molecular weight pupal cuticle proteins. The deflection of the lower precipitin band by partially purified protein 82 passes through the upper line (peak f). Thus, the low molecular weight pupal cuticle proteins share no immunlogical relatedness with high molecular weight pupal cuticle proteins. The defelction of the lower precipitin line in Figure 7, identified as peak C, originates at a position slightly more anodal than protein 56 and is, therefore, not protein 56 (protein 56 does not migrate into the antibody-containing gel under the conditions used). In addition (Figure 7, peak a), third-instar larval cuticle proteins only deflect the upper pupal cuticle protein precipitin line. This confirms the relatedness between the larval and low molecular weight pupal cuticle proteins and their lack of immunorelatedness to the high molecular weight pupal cuticle proteins.

Discussion

In a previous report, Fristrom et al. (1978) described the urea-soluble cuticle proteins of third-instar larvae of *Drosophila melanogaster*. These authors concluded, on the basis of data on amino acid compositions of purified fractions and properties of genetic variants, that the five major urea-soluble proteins were probably specified by five discrete coding regions. These conclusions were confirmed by sequence analysis of third-instar larval cuticle proteins (Snyder et al., 1982). The current report compares the urea-soluble proteins isolated from pupal and third-instar larval cuticles of *Drosophila*. The pupal cuticle proteins, like the third-instar larval cuticle proteins,

comprise a small set of electrophoretically heterogeneous polypeptides. One-dimensional fingerprints indicate that the major pupal cuticle proteins have discrete primary structures and are encoded by discrete coding sequences that differ from those for the third-instar larval cuticle proteins. This conclusion is supported by additional observations. Snyder et al. (1981) have demonstrated the absence of RNAs in pupae that are complementary to the gene sequences of third-instar larval cuticle proteins 1, 2, 3, and 4. A similar observation (unpublished results) has been made by using RNAs isolated from imaginal disks that are engaged in synthesizing pupal cuticle proteins. Furthermore, Chihara et al. (1982) have shown that variants affecting the electrophoretic migration of specific third-instar larval cuticle proteins do not alter the migration of pupal cuticle proteins.

Unlike the third-instar larval cuticle proteins, the pupal cuticle proteins fall into two discrete structural, and presumably functional, classes. One class of pupal cuticle proteins is composed of unglycosylated or poorly glycosylated polypeptides with low molecular weights (8K–25K). Despite being encoded by discrete genes, these peptides are immunologically related to each other and to the third-instar larval cuticle proteins. They are immunologically unrelated to the high molecular weight pupal cuticle proteins. With the exception of pupal cuticle protein 8, when renatured they bind—like the third-instar larval cuticle proteins—to chitin. The high molecular weight pupal cuticle proteins are glycosylated and are immunologically unrelated to the low molecular weight pupal cuticle proteins. When renatured, they have little or no affinity for chitin. Furthermore, pupal cuticle proteins 56 and 82 are synthesized later in development than the low molecular weight pupal cuticle proteins (J. Doctor, D. Fristrom, and J. W. Fristrom, unpublished results). Thus, we conclude that the low and high molecular weight pupal cuticle proteins are not only structurally unrelated but also possibly functionally unrelated as well.

We presume that in those cases where cuticle proteins belong to the same immunological family their genes have arisen by duplication and evolutionary divergence. In those cases where family members are expressed at different stages, e.g., third instar vs. pupal, we posit that divergence accomplished either or both of the following two purposes: (1) the production of proteins with properties tuned to the structural–physiological roles of the two cuticles; (2) the facilitation of temporal regulation of cuticle protein genes. In those cases where family members are expressed at the same stage, there is a third possible purpose of gene duplication: the production of increased amounts of functionally related products. The properties of cuticles have been suggested to be consequences, at least in part, of the properties of their proteins. For example, Andersen (1979) has noted a correlation between the hydrophobicity of proteins, as revealed by the amino acid compositions of total protein complements of cuticles, and the elasticity of the cuticles. If such correlations prove to be causal, they support the notion that a prime basis for divergence of cuticle protein genes between stages is to produce cuticles with differing structural properties.

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Registry No. Chitin, 1398-61-4.

References

Anderson, C. W., Baum, P. R., & Gesteland, R. F. (1973) *J. Virol. 12*, 241.

Andersen, S. O. (1979) *Annu. Rev. Entomol. 27*, 29.

Axelson, N. H., Kroll, J., & Weeke, B. (1973) *A Manual of Quantitative Immunoelectrophoresis. Methods and Applications*, Blackwell Scientific Publications, Oxford, England.

Berreur, P., Porcheron, P., Berreur-Bonnenfant, J., & Simpson, P. (1979) *J. Exp. Zool. 210*, 347.

Boeseken, J. (1949) *Adv. Carbohydr. Chem. 4*, 189.

Borst, D. W., Bollenbacher, W. E., O'Connor, J. D., King, D. S., & Fristrom, J. W. (1974) *Dev. Biol. 39*, 308.

Campbell, D. H., Garvey, J. S., Cremer, N. E., & Sussdorf, D. H. (1963) in *Methods in Immunology*, W. A. Benjamin, New York.

Chihara, C. J., Silvert, D. J., & Fristrom, J. W. (1982) *Dev. Biol. 89*, 379.

Cleveland, D. W., Fischer, S. G., Kirshner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102.

Downe, A. E. R. (1962) *Can. J. Zool. 40*, 957.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Fraenkel, G. (1935) *Proc. R. Soc. London, Ser. B 108*, 1.

Fraenkel, G., & Rudall, K. M. (1947) *Proc. R. Soc. London, Ser. B 134*, 111.

Fraenkel, G., & Bhaskaran, G. (1973) *Ann. Entomol. Soc. Am. 66*, 418.

Fristrom, J. W., Hill, R. J., & Watt, F. (1978) *Biochemistry 17*, 3917.

Fukushi, Y., & Seki, T. (1965) *Jpn. J. Genet. 40*, 203.

Gold, A. M. (1967) *Methods Enzymol. 11*, 706.

Hackman, R. H. (1955a) *Aust. J. Biol. Sci. 8*, 83.

Hackman, R. H. (1955b) *Aust. J. Biol. Sci. 8*, 530.

Hackman, R. H., & Goldberg, M. (1978) *Insect Biochem. 8*, 353.

Hodgetts, R. B., Sage, B., & O'Connor, J. D. (1977) *Dev. Biol. 60*, 310.

Hood, L., Campbell, J. H., & Elgin, S. C. R. (1975) *Annu. Rev. Genet. 9*, 305.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Locke, M. (1974) in *The Physiology of Insecta* (Rockstein, M., Ed.) Vol. VI, p 123, Academic Press, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Lundblad, A., Nilsson, B., Norden, N., Svensson, S., Ockerman, P., & Jolly, R. (1975) *Eur. J. Biochem. 59*, 601.

Madhavan, M. M., & Madhavan, K. (1980) *J. Embryol. Exp. Morphol. 60*, 1.

Mitchell, H. K., & Mitchell, A. (1964) *Drosoph. Inf. Serv. 39*, 135.

Narasimhan, S., Harpaz, N., Longmore, G., Carver, J. P., Grey, A. A., & Schacter, H. (1980) *J. Biol. Chem. 255*, 4876.

Poodry, C. A. (1980) in *The Genetics and Biology of Drosophila* (Ashburner, M., & Wright, T. R. F., Eds.) Vol. 2d, p 443, Academic Press, London.

Reichlin, M., Nisonoff, A., & Margoliash, E. (1970) *J. Biol. Chem. 245*, 947.

Roseland, C. R., & Schneiderman, H. A. (1979) *Wilhelm Roux's Arch. Dev. Biol. 186*, 235.

Snyder, M., Hirsh, J., & Davidson, N. (1981) *Cell (Cambridge, Mass.) 25*, 165.
Snyder, M., Hunkapiller, M., Yuen, D., Silvert, D., Fristrom, J., & Davidson, N. (1982) *Cell (Cambridge, Mass.) 29*, 1027.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Weitzman, S., Scott, V., & Keegstra, K. (1979) *Anal. Biochem. 97*, 438.
Whitten, J. (1968) in *Metamorphosis* (Etkin, W., & Gilbert, L., Eds.) p 43, Appleton-Century-Crofts, New York.

A Specific Subunit of Vitellogenin That Mediates Receptor Binding†

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ABSTRACT: Vitellogenin, an estrogen-induced serum protein synthesized in the liver, is composed of two M_r 250K polypeptides. It is specifically transported by a receptor-mediated endocytic process into the developing oocytes of virtually all oviparous animals. Following endocytosis, in the chicken, vitellogenin is specifically processed to yield several smaller products including the phosvitins (PV) and the lipovitellins (LV). These products are then stored within the oocyte until they are degraded during embryogenesis to provide nutrients for the developing embryo. Direct binding studies using iodinated vitellogenin demonstrate that vitellogenin binds to isolated oocyte membranes with a K_D of 2.5 μM. Competition studies indicate that PV is a competitive inhibitor of vitellogenin binding. This leads us to propose that the PV portion of the circulating vitellogenin molecule mediates binding and uptake. Direct binding studies using iodinated PV show that PV binds to isolated oocyte membranes with a K_D of 2.4 μM. Competition studies also demonstrate that 3.1 μM vitellogenin inhibits 50% of control ^{125}I-PV binding, but IgG and bovine serum albumin at concentrations up to 10 μM have no effect on ^{125}I-PV binding. Another series of competition experiments using a constant amount of vitellogenin and increasing amounts of ^{125}I-PV indicate that vitellogenin acts as a competitive inhibitor of PV binding and has a K_I of 2-3 μM. These results support our hypothesis that the receptor which mediates vitellogenin binding and uptake recognizes determinants on the PV portion of the native vitellogenin molecule.

Selective protein transport, mediated by specific receptors in association with coated pits and coated vesicles, is a fundamental cellular process (Roth & Porter, 1964; Goldstein et al., 1979; Anderson & Kaplan, 1983). The vital role of this receptor-mediated endocytic process is particularly manifested during reproduction. The selective transport of maternal immunoglobulins into the offspring provides the newborn with passive, maternally derived immunity until it becomes immunocompetent (Brambell, 1970). In addition to IgG, the specific transport of other maternal proteins is also essential for successful reproduction. A particularly graphic example occurs in all oviparous animals, where vitellogenin is selectively incorporated into the developing oocytes of these animals. Vitellogenin is synthesized in the liver in response to estrogen, carried by the circulation to the ovary, and incorporated into the developing oocyte by receptor-mediated endocytosis (Bergink & Wallace, 1974; Paterson et al., 1962; Schjeide et al., 1963; Wallace & Dumont, 1968). After its internalization, vitellogenin is specifically processed to yield phosvitin (PV) and lipovitellin (LV) (Deeley et al., 1975; Christmann et al., 1977). Two species of phosvitin have been identified from egg yolk with M_r's of 28K and 34K (Clark, 1970). Following fertilization, vitellogenin is degraded to provide nutrients for the developing embryo.

The developing chicken oocyte provides an excellent model system in which to study receptor-mediated endocytosis. During the last 5 days of development, a single oocyte will internalize up to 1 g of protein/day. Vitellogenin makes up the major portion of this transported protein. We (Yusko & Roth, 1976; Yusko et al., 1981) and others (Wallace & Jarad, 1976; Opresko et al., 1980; Engelmann, 1979) have shown that the first step in vitellogenin uptake is the receptor-mediated binding of vitellogenin to the oocyte plasma membrane surface. In this report we describe experiments that explore the binding properties of the vitellogenin receptor and show that "in vitro" the vitellogenin receptor recognizes determinants on its phosvitin moiety.

Experimental Procedures

Materials

Bovine serum albumin, chicken serum albumin, and phosvitin were purchased from Sigma Chemical Co. Sepharose CL-6B was obtained from Pharmacia. Acrylamide, bis(acrylamide), Enzymobeads, Bio-Gel P-10, and Bio-Gel A-1.5m were from Bio-Rad Laboratories. DE-52 ion-exchange resin was from Whatman. All other chemicals were of reagent grade and were purchased from commercial sources. Live white Leghorn laying hens, roosters, and developing oocytes were obtained from a local slaughter house.

Chicken IgG, kindly provided by Dr. John Soper, was purified from egg yolks by a modification of the method of Bernardi & Cook (1960) (Linden & Roth, 1978).

All experimental procedures were carried out in an incubation buffer consisting of 0.01 M 2-(*N*-morpholino)ethanesulfonic acid (MES),[1] pH 6.0, 0.14 M NaCl, 5 mM KCl, 0.83

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[1] Abbreviations: IB, incubation buffer; IB-BSA, incubation buffer plus 10 mg/mL bovine serum albumin; PV, phosvitin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; LV, lipovitellin; M_r, molecular weight; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride.

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