

## Structural Proteins of Sarcophagid Larval Exoskeleton

COMPOSITION AND DISTRIBUTION OF RADIOACTIVITY DERIVED FROM [7-<sup>14</sup>C]DOPAMINE\*

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In the cyclorrhaphid flies, exoskeletal proteins from the last larval instar cross-link by arylation and glycosylation to form the sclerotized puparial case. Cuticular proteins from maggots killed just prior to tanning were resolved into 21 soluble components by isoelectric focusing and sodium dodecyl sulfate-polyacrylamide electrophoresis. Isoelectric points ranged from pH 4.5 to 6.0, molecular weights were distributed between  $M_r = 16,000$  and 24,000. Aspartic and glutamic acids, glycine, serine, valine, and lysine were abundant in all the proteins while sulfur-containing residues were uniformly absent. Heterogeneity was manifest among  $\text{NH}_2$  termini of the soluble fractions, while the insoluble chitin-linked protein showed only aspartic acid in this position. The sclerotized matrix was assembled by a concerted bridging of protomers without accumulation of di-, tri-, or higher n-mers in the urea-soluble fraction. This mechanism was also favored by uniform distribution of the bridge precursor, [7-<sup>14</sup>C]dopamine, among the individual larval protomers including the polypeptide bound to chitin. Following administration of isotopic catecholamine 2 to 10 h prior to sclerotization, unbridged larval cuticle retained 3% of the radioactivity, puparial and adult integument 7% and 18%, respectively. Proteolytic digestion afforded labeled peptides with molecular weights in register with the degree of cross-linking. Nonradioactive larval proteins did not incorporate labeled dopamine and exchange incubation of labeled proteins with nonisotopic precursor failed to diminish recoveries of <sup>14</sup>C. Since protein synthesis was low as assessed by minimal incorporation of [<sup>3</sup>H]leucine, metabolites derived from dopamine may have been added after translation in the course of presclerotical activation of the polypeptides destined for cross-linking.

The transformation of unconjugated soluble polypeptides and chitin-bound protein of maggot procuticle to the hard pigmented puparial sheath coincides with uptake of phenolic intermediates derived from tyrosine, increased covalent bonding between chitin and protein, and reduction in water content (1-3). In the higher Diptera, hardening does not require major synthesis of new structural protein or polysaccharide. Thus the cyclorrhaphid system serves as a good model for the study of tanning (4, 5). The molecular weight of the soluble proto-

mers destined for polymerization is in the range of 12,000 to 26,000, isoelectric points lie between pH 4 and 6, and 60% of the amino acid titer consists of aspartic acid, glutamic acid, glycine, valine, and lysine (6). In contrast to the larval integument, the sclerotized puparium is resistant to proteolytic or chitinolytic digestion, to chemical cleavage with NBS,<sup>1</sup> sodium-ammonia, or alkaline borohydride, and to dispersal in chaotropic solvents containing urea, SDS, or alkyldiamines (2, 7-10). The scheme currently favored for assembly of the arylated matrix features addition of polypeptides to the ring or side chain of catecholamines or phenolic acids. Linkage is affected via  $\epsilon$ -amino of lysine or unidentified  $\text{NH}_2$  termini. Presumably, the benzenoid precursor is activated affording a transient quinone prior to conjugation since molecular oxygen and a tissue-specific polyphenol oxidase are also required for polymerization (1). The order of assembly of polypeptide protomers also remains to be established in the Cyclorrhapha. Although long lived di- and trimer intermediates have been described from lower orders of insects, the occurrence of partially condensed n-mers has not been examined in the model maggot-puparial system (11, 12).

Enhancement of chitin-protein bonding is also conjectural since chemical evidence is limited to increased recoveries of peptidochitodextrins from chitinase-protease limit digests of puparial integument treated with NBS (2). Post-translational modification of protein and polysaccharide prior to complex formation is likely from evidence provided by tissue analysis and studies with cell-free systems. Isolated cuticle proteins from larvae fail to polymerize in the presence of homologous polyphenol oxidase,  $\text{O}_2$ , dopamine, and a wide variety of activators (6).  $\beta$ -Alanine has been recovered from puparial hydrolysates but not from untanned precursor proteins (13). As a preliminary to the investigation of the mechanisms of sclerotization, *per se*, this contribution describes the isolation and partial characterization of the unmodified larval structural proteins together with observations on the fate of labeled dopamine administered prior to the onset of hardening. A preliminary account of this work has appeared in abstract form (14).

### MATERIALS AND METHODS AND RESULTS<sup>2</sup>

<sup>1</sup> The abbreviations used are: NBS, N-bromosuccinimide; PMSF, phenylmethanesulfonyl fluoride; PTU, phenylthiourea; SDS, sodium dodecyl sulfate; dopa, 3,4-dihydroxyphenylalanine.

<sup>2</sup> Portions of this paper (including "Materials and Methods," "Results," Figs. 1 to 3, and Tables I to IV) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-2193, cite author(s), and include a check or money order for \$5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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## DISCUSSION

Borate-soluble proteins from the procuticle of *Sarcophaga bullata* have been resolved into 21 homogeneous polypeptides as assessed by isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Attempts to demonstrate heterogeneity by column chromatography on DEAE-cellulose or by electrophoresis on urea gels failed to reveal secondary components within the individual focused bands. Evidence favoring purity of the individual isolates, to be reported in detail in a subsequent publication, has been obtained by identification of a single NH<sub>2</sub> terminus for five of the components (24). The NH<sub>2</sub> termini were not identical, however. Most soluble cuticle proteins from arthropods range from  $M_r = 16,000$  to 24,000 and are usually rich in acidic amino acids, glycine, valine, and lysine with somewhat lesser amounts of alanine and serine. The minor variations in these six residues impose heterogeneity of a low order to this large group of functionally related polypeptides. Hackman (25) ascribed this variation to genetic polymorphism. Fristrom *et al.* (26) examined integumental proteins of *Drosophila* for separate coding regions and for individual variants in natural populations; neither criterion was in accord with this proposal. *Sarcophaga*, *Calliphora*, and *Lucilia* from the same suborder of Diptera have cuticle proteins similar with respect to isoelectric points, molecular weight range, ratios of hydrophobic to hydrophilic residues, and absence of sulfur-containing amino acids. *Drosophila*, another cyclorrhaphid, may lack several proteins common to the other two species (26). Similarities in amino acid composition suggest regions of sequence homology, but the variation is sufficient to restrict putative sequence identities to short internal stretches. Restricted homology has been reported for feather keratins from chick embryo (27) and may be common to cross-linked structural matrices comprised of many bridged protomers. The identification of hydroxylysine in Protein 12 suggests that remnants of the collagenous cuticular basement were also soluble in borate-SDS; hydroxyproline and bound hexose were absent, in common with other insect collagens (28). Proteins 2, 4, 5, 16, and 19 contained single residues of  $\beta$ -alanine, an indicator of the onset of pupariation in this species (29). This amino acid is absent from the cuticle of less advanced stages, suggesting that these proteins are either deposited early during the postfeeding stage or represent an unusual post-translational modification of existing protein. On the basis of the variability in amino acid composition, none of the remaining 14 proteins could serve as precursors for the modified polypeptides by addition of a single residue of  $\beta$ -alanine. In some dipterans and lepidoterans,  $\beta$ -alanine is a constituent of melanoprotein rather than of support substance. Proteins modified by the insertion of  $\beta$ -alanine, therefore, may constitute putative chromoproteins rather than soluble presclerotol initiators of cross-linking (30, 31). Since radioactivity of the  $\beta$ -alanine-enriched components was of the same order as the other proteins acquiring label from [7-<sup>14</sup>C]dopamine (Table III), it is probable that these five entities were modified at the same time as companion proteins, all subsequently participating in the formation of the sclerotized matrix and coloration of the puparium (32, 33).

Protomers ranging from  $M_r = 16,000$  to 24,000 would afford di- and trimer intermediates from  $M_r = 32,000$  to 70,000 in the course of step-wise cross-linking to sclerotized products. Soluble intermediates were not detected on urea gels (Fig. 2); hence partially tanned structural members either do not exist or accumulate elsewhere than the urea- or borate-soluble fractions. Provided the assumed intermediates occur in measurable amounts, inability to observe their presence may originate in reduced staining following arylation, in declining solubility, or to extensive proteolysis immediately preceding

polymerization. However, a search for partially polymerized materials of higher molecular weight on molecular sieves gave no evidence of the presence of larger species with uv-absorption as the criterion of assembly (6). The ubiquity of the dopamine metabolite within the individual larval isolates (Fig. 2) is also in keeping with a concerted assembly rather than the preliminary joining of specific proteins. When borate-extracted larval or sclerotized cuticle was exposed to 0.25 M NaOH in an atmosphere of N<sub>2</sub>, low levels of material were solubilized that were excluded from 10% gels, indicative of a molecular mass of 200,000 daltons or greater. Since strong base catalyzes nonspecific cross-linking of tyrosyl and related substituents even in an oxygen-poor environment, evidence favoring these larger oligomers requires further investigation. On the other hand, diversity of function between structural proteins and an ordered progression of cross-linking may be indicated by the wide variation in amino acid composition, particularly for  $\beta$ -alanine. Following modification by a metabolite of dopamine (Table III), addition of  $\beta$ -alanine to a number of the proteins may initiate concerted bridging.

One-third of the integumental protein fails to dissolve in borate, urea, or cold dilute sodium hydroxide, suggesting covalent linkage to chitin. Although initially unresponsive to digestion by polysaccharases, this fraction is susceptible to proteolysis with the generation of glutamic acid-rich peptidochitodextrins accessible to chitinase (2, 7). The chitin-peptide bonds are not cleaved by NBS, sulfite addition, alkaline borohydride, Birch reduction, nitrous acid, or dilute sodium hydroxide under conditions conducive to splitting of aspartamido or ester linkages, suggesting atypical joining of the two macromolecules (9, 32-34). The recovery of a single NH<sub>2</sub> terminus, aspartic acid, from the intact chitin-protein complex followed by equivalent yields of the second (valine) and third (alanine) residues introduces several possibilities concerning the distribution of chitin and protein within this complex. Only a single protein species may be involved, or, if the protein component is heterogeneous, companion proteins may be blocked at this locus. The possibility exists that chitin *per se* or an unidentified bridging group occupies unreactive NH<sub>2</sub> termini rather than the conventional acyl or pyroglutamyl groups in blocked proteins. On the other hand, occupation of the COOH terminus seems probable, since prolonged incubation with three carboxypeptidases failed to release amino acids from this position. In view of the resistance of the chitin-protein linkage to alkali, however, acylation of the C3 or C6 hydroxyl of N-acetylglucosamine may be unlikely. Occasional substitution of amino N of the hexosamine in place of N-acetyl remains a possibility.

The incorporation of <sup>14</sup>C from the side chain of dopamine during the larval stage when protein synthesis is depressed and catabolism of C7 and C8 of the labeled precursor to <sup>14</sup>CO<sub>2</sub> is barely detectable suggests presclerotol activation of the soluble unpigmented proteins. The protein adducts are not formed *in vitro*, either in the presence or absence of homologous cuticle polyphenol oxidase, a situation in accord with a requirement for modification of the monomers prior to incorporation of the dopamine-derived metabolite (6). The labeled proteins and peptides did not bind to affinity columns specific for *o*-diphenols either before or after acid hydrolysis, and this property was unchanged by initial treatment with sodium borohydride. These properties introduce the possibility that a portion of the phenol or quinone functions are modified by *O*-alkylation or by the generation of an open chain structure.

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SUPPLEMENT TO  
STRUCTURAL PROTEINS OF SARCOPHAGID LARVAL EXOSKELETON  
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# MATERIALS AND METHODS

**Extraction of Structural Proteins** - *Sarcophaga bullata* were reared on horse meat to the postfeeding stage and synchronized by immersion prior to dissection and administration of isotope [15]. Batches of maggots (20 g) were washed with water and chilled in ice prior to disruption in 100 ml of 50 mM acetate buffer, pH 5.5, containing 0.2 mM PMSF and saturated with PPU to inhibit proteolytic enzymes and polyphenol oxidases, respectively. All subsequent washings and extractions included these protective agents. The suspension was homogenized at 27°C, at half maximum speed and twice at full speed in a Virtis Omulizer and the cuticle isolated by sieving through cheesecloth. The tissue was washed with 3 l of the same buffer and suspended briefly in water prior to transfer to 500 ml of extraction medium. The cuticle preparations were examined at 100X for remnants of viscerae, and blending was repeated until pure cuticle was evident. Soluble structural proteins were extracted with 2% (v/v)  $\text{Na}_2\text{HPO}_4$  containing 0.5% SDS, and 10 mM mercaptoethanol adjusted to pH 9.1. The suspension was stirred at 27°C for 18 h, the tissue collected by decantation of the supernatant solution, and re-extracted with 250 ml of the same solvent. The extracts were combined and clarified by sedimentation at 10,000 X gaw and the supernatant solution adjusted to pH 4.5 with 10 M acetic acid. After 12 hours at 2°C the precipitate was removed by centrifugation at 15,000 X gaw and discarded. The supernatant was brought to 0.5% (v/v) ethanol by the gradual addition of the chilled reagent. Following agitation for 18 h, the precipitate was collected by sedimentation at 20,000 X gaw and dissolved in 50 mM  $\text{Na}_2\text{citrate}$ -1M KCN. Following dialysis against 10 vol of the same buffer for 24 h with 2 changes, the preparation was lyophilized and stored under vacuum at -20°C. Proteins soluble in urea were prepared from the borate-SDS extracted residue by suspension in 8 M urea (recrystallized containing 50 mM K-phosphate, pH 8.0. Following urea treatment, the insoluble polypeptide associated with the chitin was washed with 20 mM ascorbic acid and water and lyophilized (Fraction CH-1). Urea extracts examined for tanning oligomers were prepared from cuticles previously extracted with 50 mM acetic acid with the borate step omitted. For determination of radioactivity, urea and borate-SDS treated integuments were washed with water and extracted with 0.25 M NaOH under a stream of  $\text{N}_2$  following which the skins were washed and dried. Proteins from the white puparium, a preliminary stage in sclerotization, and from partially or fully tanned material were prepared from cuticles scraped free of adhering tissues and subjected to the same extraction procedures.

**Polycrylamide Gel Electrophoresis** - Isoelectric focusing was performed at 5°C in cylinders (0.5 X 10.5 cm) containing 7% acrylamide with 0.04 M Ampholine, pH 4-6 (10% the upper AB) per ml of gel. The upper cathode chamber contained 0.02 M NaOH, the lower chamber contained 0.01 M  $\text{H}_2\text{PO}_4$ . Each tube accommodated 400  $\mu\text{g}$  of cuticle protein. After pre-focusing, current was applied at 3.5 mA per tube for 6 h. Bands for identification and photography were visualized by staining with Coomassie Blue R. Band display for chemical analysis was accomplished by four immersions in 0.7 saturated  $(\text{NH}_4)_2\text{SO}_4$  at 2°C for removal of ampholine. Each component was isolated by sectioning the gel, and pooled with proteins of identical mobility. The gel cylinders were crushed prior to elution of the proteins with 50 mM Tris buffer, pH 8.0. The eluate was passed through a 0.22 filter (Millipore) dialyzed and lyophilized. Molecular mass and purity of the proteins separated by isoelectric focusing were assessed by electrophoresis of 50  $\mu\text{g}$  samples following dissociation with 0.1% SDS and mercapto-ethanol with the running and lower reservoir buffers adjusted to pH 9.20 [16]. Formation of oligomers was examined in combined separation and dissociation systems with 8 M urea added to the running gel buffer. Standards consisted of proteins of molecular masses as follows: Insulin, B chain (3,300 daltons); cytochrome C (11,700 daltons); lysozyme (14,300 daltons); soy bean trypsin inhibitor (21,500 daltons); BPP-trypsin (23,300 daltons); chymotrypsinogen (25,700 daltons); carboxyl esterase (30,000 daltons); ovalbumin (43,000 daltons); bovine serum albumin (68,000 daltons); and phosphorylase B (94,000 daltons).

**Labelling of cuticle proteins** - Synchronized third stage post-feeding larvae were injected through the spiracular plates with 1  $\mu\text{l}$  of 0.1 M NaCl containing 1 mM  $\text{K}_2\text{HPO}_4$  (pH 6.9) by means of a 5  $\mu\text{l}$  syringe. Each larva received 0.8  $\mu\text{Ci}$  of  $[7\text{-}^{14}\text{C}]$  dopamine, 36  $\mu\text{Ci}/\text{mmol}$ , (New England Nuclear) while immobilized by chilling. Dopamine synthesis was assessed by simultaneous administration of 1-[4,5- $^3\text{H}$ ] leucine. Animals were warmed to 20°C and transferred to paper towels, those exhibiting loss of hemolymph were discarded. Following additional synchronization by immersion for three hours, the larvae were placed on wet towelling to prolong the immature stage or transferred to dry wood shavings for pupariation. Moist individuals retaining the maggot body conformation between two and ten hours were classified as immatures and processed for larval cuticle as described above. The dry puparium was sacrificed when the white, amber or dark puparial stages were attained. Following isolation by gel electrophoresis, proteins were extracted from the gel and quantified on the basis of amino acid recoveries from hydrolyzates. Loosely bound radioactivity was removed from the proteins by solution in 5  $\mu\text{l}$  of 0.2 M dopamine adjusted to pH 6.0 at 10°C for 2 h. After exchange, the proteins were dialyzed against 2 mM  $\text{NH}_4\text{HCO}_3$  and lyophilized. Radioactivity was determined with Aquasol II scintillation fluid (New England Nuclear) in a Packard Model 3302 scintillation spectrometer.

**Amino Acid Composition** - Protein samples were dialyzed against 30 mM  $\text{NH}_4\text{HCO}_3$ , lyophilized and hydrolyzed in sealed, evacuated tubes at 115°C for 16 h. Amino acids routinely found in protein hydrolyzates were liberated by constant boiling HCl (5.8 M) fortified with 400  $\mu\text{g}/\text{ml}$  phenol. Acid was removed by evaporation or ion pairing with trioctylamine [17]. Tryptophane, cysteine, phenols and radioactive metabolites not reacting with ninhydrin were protected with mercuron or indole-fortified sulfuric acids. Mercapto-ethanesulfonic acid (3 M) or methanesulfonic acid (4 M) containing 0.2% 2-amino-ethyl indole were added at a level of 1  $\mu\text{l}/\text{mg}$  of protein. Recovery of tryptophane, methionine and cysteine from model proteins exceeded 80% [18,19]. Cysteine was converted to  $\alpha$ -keto-cysteine with dithionitro and an excess of tetrathionate. Following hydrolysis, 4 M NaOH was added to pH 2 and the amino acid composition was determined on a Beckman 119-C instrument. Samples were analyzed in triplicate. Amino termini were determined by manual Edman degradation [20].

**Analysis of Labelled Proteins** - Unresolved larval and puparial proteins soluble in borate buffer were digested serially with *Streptococcus aureus* protease V8 (Miles Laboratories), Subtilisin BPN' (Nagase Ltd.), Pronase B (Calbiochem), TPCK-trypsin (Sigma Chemical Corp.) at pH 7.5 in 5 mM  $\text{NH}_4\text{HCO}_3$  containing 0.1 mM  $\text{Ca}^{2+}$ . The digest was adjusted to pH 2 for a final treatment with pepsin (Worthington Biochemical Co.). Enzymes were added at a substrate to protease ratio of 50. Digests were heated to 100°C for 5 minutes and clarified by centrifugation between addition of each enzyme. The samples were chromatographed on Bio-Gel P-2 at 55°C in 0.1 M acetic acid. These conditions favor retardation of aromatic amines by absorption to the polyacrylamide matrix [21]. Fractions of 5  $\mu\text{l}$  were examined for radioactivity and uv-absorbance. Affinity chromatography for 1,2-diols was performed in 1 X 3 cm columns packed with N-(N'-m-dihydroxyborylphenyl) succinyl] amino ethyl cellulose [22].

## RESULTS

**Solubility and Composition of Cuticle Extracts** - Of the total cuticular larval polypeptide 28.5% was recovered by extraction with borate and precipitation with 20% ethyl alcohol. The protein remaining associated with the residual chitin represented 36.2%. Less than 5% of the bound protein was released by additional treatment with 8 M urea or 0.25 M NaOH. Omission of the borate step resulted in 60.4% of the protein to the urea extract but the preparation declined in solubility with time and was less suitable for isoelectric focusing. The intermediate white puparial stage showed substantially the same properties, but the dark puparium declined in solubility on sclerotization. Amber colored and black puparia released 22.4% and 14% of the dry weight into borate, respectively. When the borate step was omitted, 23.2% of the darker puparium was solubilized by urea. Not considered in values for larval cuticle are the minor quantities (4%) precipitated from borate by acidification to pH 4.5 and the fraction remaining in solution after addition of alcohol (2%). An amino acid composition resembling that of dipteran muscle characterized the latter fractions. The efficiency and specificity of the disruption procedure for bulk samples was assessed by extraction and gel electrophoresis of two groups of larvae, one subjected to hand dissection of individual larvae, and one to bulk processing. No differences could be detected.

The borate-soluble material required storage at -20°C under reduced pressure in the presence of mercaptoethanol vapors to maintain solubility for periods in excess of one month, routinely electrophoresis was performed within ten days of extraction. Examination of freshly prepared cuticle extracts for bound aldoses, pentoses and amino sugars revealed no detectable carbohydrate in 5 mg of protein. The assays were sensitive to 0.05  $\mu\text{mol}$  of each type of sugar. Extraction of unresolved larval proteins with ether and chloroform did not reduce the dry weight of the sample. Intact and hydrolyzed proteins were subjected to transesterification with 2% in methyl alcohol to identify presumptive bound lipids. Gas-liquid chromatography failed to reveal fatty acid ethyl esters.

**Gel Electrophoresis of Cuticle Proteins** - Isoelectric focusing over the range pH 4-6 resolved twenty-one components of a high degree of purity (Fig. 1). Electrophoresis in urea or in non-dissociating solvents revealed approximately eight major and four minor components in 1% gels, all corresponded to one or more of the focused components. The patterns were extremely reproducible from run to run and with populations of *Sarcophaga bullata* of diverse origin. Band distribution was not influenced by seasonal fluctuations in the development rate of the larvae or fecundity of the adults. When proteins recovered from the acidic extremes of the pH range, 4.50-4.90 were focused on ampholines poised at pH 3.0-5.0, no changes in mobility or heterogeneity were evident. Similar results were observed when pH 5.5-5.9 fractions were subjected to focusing over the range pH 5.0-7.0.

**Properties of Focused Components** - Although relative amounts of the twenty-one proteins varied over a wide range, distribution was even with respect to isoelectric points, with approximately eight components per 0.5 pH unit (TABLE I). Molecular masses ranged from 16,000 to 24,000, a remarkably narrow span. Electrophoresis on 1% gels in the absence of SDS failed to detect non-covalent aggregation. Although resolution was limited, all the proteins remained in the area of the gels characteristic of acidic polypeptides of molecular weight 11,000 to 26,000 daltons. Proteins 8 and 19 were present in highest titers followed by components 3, 6, 7, 12, 15 and 10. The possibility that the abundance of distinct structural polypeptides and the microheterogeneity was an artifact of limited proteolysis was investigated by sample preparation in the presence and absence of PMSF and by pressing of fresh and aged cuticle extracts. No significant enhancement of low molecular weight components at the expense of larger entities was observed. Proteins harvested from populations sacrificed 36 hours earlier were identical in yield and physical properties.

In accord with migration of the proteins during focusing at pH 4-6, aspartic and glutamic acids were the predominant charged residues in all the proteins, exceeding basic residues by a factor of 2.0 to 2.8 (TABLE II). Serine, glycine, valine and lysine were present in relative abundance, in all cases polar residues exceeded non-polar. Noteworthy variation featured recoveries of histidine, tyrosine, and  $\beta$ -alanine, with sulfur amino acids uniformly absent. Treatment with cyanogen bromide failed to generate new termini and mobility was unchanged if reduction with mercaptoethanol was omitted from the SDS gel procedure. Considerable diversity was encountered with respect to the amino termini of proteins 3, 5, 12, 15 and 19. Both polar and non-polar residues were recovered from this position. In all cases the ratio of acidic to basic amino acids generally reflected the focusing patterns, but did not coincide rigorously with the isoelectric point. The values for aspartic and glutamic acids do not consider amides which occur in substantial levels as judged by Edman degradation of individual proteins. Considerable tertiary structure is retained by the mixture of cuticle proteins as evidenced by a ratio approaching unity of buried to exposed tyrosyl residues as assessed with acetylmaldoxole (23). The polypeptide associated with chitin released only aspartic acid at the N-termini when subjected to the Edman procedure. The second cycle afforded valine and the third alanine in molar equivalents to the N-terminal residue. Combinations of carbonylpeptidase A, B and Y failed to release amino acids from the C-terminus suggesting occupation of this site by linkage directly to polysaccharide or to a bridge solely unreactive to ninhydrin or the phenol-sulfuric acid test for neutral sugars.

**Soluble Proteins of Sclerotized Integument** - Gel electrophoresis of developing puparia under conditions favoring dissociation provided a means of distinguishing stepwise from concerted assembly of cross-linked proteins. The recovery of long-lived dimers and trimers with molecular weights expressed as simple integers of the monomers would support the contention that these three components are present initially with subsequent polymerization to the insoluble sclerotized matrix. On the other hand a concerted mechanism or a pathway where intermediates are present for very brief intervals and in low concentration would be manifest by the absence of material in the 35,000 to 70,000 dalton region of the gels. These alternatives were examined by gel electrophoresis of urea extracts of successive prepupal, white puparial and amber colored stages. It can be seen in Fig. 2 that no evidence of low molecular weight intermediates was discerned when extracts of sclerotized populations were compared with earlier stages of development. The gels were run at a protein concentration where a 3% conversion to n-mer could be detected assuming uptake of stain equal to that of a monomer. Overloading of gels or molecular sieving on Bio-Gel P-150 (6) on a scale 10-fold in excess of electrophoretic capacity did not alter these findings.

**Distribution of Radioactivity from  $[7\text{-}^{14}\text{C}]$  Dopamine** - In the course of investigations on the fate of  $[7\text{-}^{14}\text{C}]$  dopamine and  $[4,5\text{-}^3\text{H}]$  leucine during sclerotization, the former but not the latter isotope accumulated in cuticle proteins extracted from maggots four to ten hours after treatment. Although proteins labelled with  $[^3\text{H}]$  leucine were recovered from the hemolymph and viscerae, no cuticle component exceeded 95 dpm/mg (2 dpm/nmol). The enhanced oxidative activity accompanying metamorphosis did not include C7 of dopamine, less than 1% of the radioactivity administered was metabolized to respiratory  $^{14}\text{CO}_2$  during 15 hours. Distribution of radioactivity among the individual proteins was assessed by focusing, excision and elution of the bands from the gels, determination of yield by gravimetry and scintillation counting of the purified components. It is evident that all bands incorporated radioactivity from precursor to approximately the same degree (TABLE III). The  $^{14}\text{C}$ -labelled material was not removed by repeated washing with unlabelled dopamine. Incubation in K-phosphate (pH 6.5) of unlabelled cuticle proteins with  $[7\text{-}^{14}\text{C}]$  dopamine at a level of 1  $\mu\text{Ci}/\text{mg}$  protein followed by dialysis against dopamine afforded quantitative recovery of isotope in the diffusate and unlabelled proteins in the retentate.

Partition of isotope among the borate, urea and sodium hydroxide extracts and the insoluble residue (TABLE IV) showed minor differences in specific activities within the larval stage. Total recoveries from cuticle strongly favored the borate-SDS compartment, however. Insoluble proteins cross-linked via arylation and glycosylation sequestered threefold as much isotope per unit weight in the amber colored puparium, with little difference in specific activities between the soluble fractions. With respect to total incorporation into all cuticle fractions, whereas 18% of the total radioactivity was bound to the insoluble larval chitin-protein complex, 61% was rendered insoluble by puparial sclerotization and 53% in the fully hardened adult.

**Metabolites of  $[7\text{-}^{14}\text{C}]$  Dopamine in Enzyme Digests of Larval and Puparial Proteins** - Molecular sieve chromatography of 1  $\mu\text{Ci}$  digests from unsclerotized larval populations was tanned material labelled with  $[7\text{-}^{14}\text{C}]$  dopamine revealed marked diversity with respect to molecular weight and absorption spectra of the radioactive fragments. Partial hydrolysis by serial digestion with six proteases, followed by chromatography on Bio-Gel P-2 under conditions favoring delayed elution of aromatics [21] resulted in distinct patterns of uv-absorption, and  $^{14}\text{C}$  recovery (Fig. 3). In the case of larval cuticle, the bulk of the radioactivity co-chromatographed with authentic markers of 100-200 daltons. Puparial material, on the other hand, confined radioactivity to the exclusion volume coinciding with the emergence of non-aromatic markers of about 2,000 daltons. In contrast to acid hydrolyzates, the radioactive components from proteolytic digests of amber puparia were virtually transparent at 430 nm, differing in this criterion from the  $^{14}\text{C}$ -labelled larval material subjected to enzymic hydrolysis. The availability of an affinity support procedure specific for 1,2-diols [22] facilitated examination of the borate-soluble proteins for the  $\alpha$ -diol functions. The column bound dopamine, pro-catecholic acid and dopa avidly but retained less than 2% of the radioactivity present in puparial digests. Prior reduction with sodium borohydride for conversion of quinones to phenols did not alter this finding.

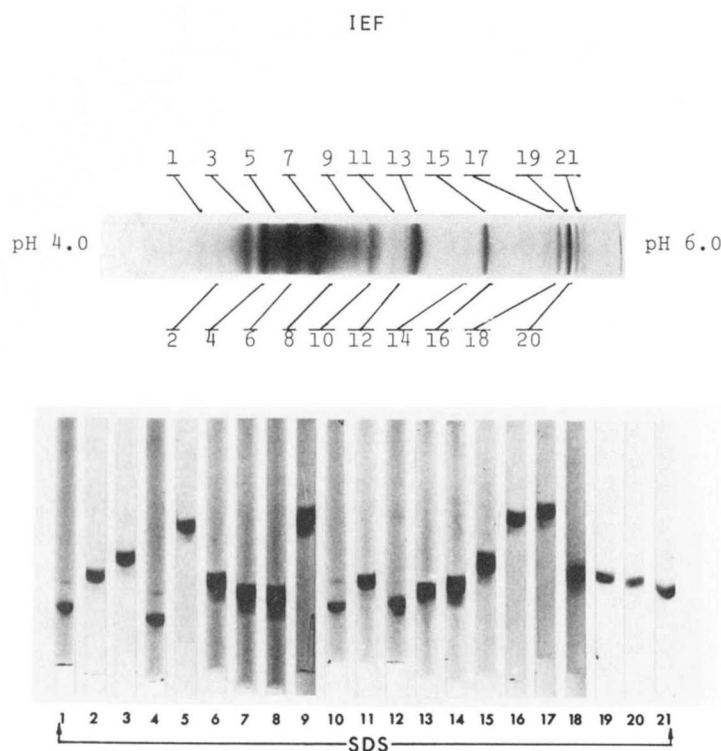


Fig. 1. SDS-polyacrylamide electrophoresis of cuticle proteins resolved initially by isoelectric focusing. Lane IEF, borate-solubilized, alcohol precipitable proteins (500  $\mu$ g) were focused in 7% acrylamide with carrier ampholytes selected to give a pH gradient of 4.0 to 6.0. Bands stained with Coomassie Blue R. For SDS-electrophoresis, focused bands were visualized by immersion in 0.7 saturated  $(\text{NH}_4)_2\text{SO}_4$ , the gel sectioned and components with similar mobility pooled for final resolution. Lanes 1 to 21, purity was assessed by electrophoresis on SDS-polyacrylamide gels (10%) loaded with 40 to 100  $\mu$ g protein. Molecular weights determined on separate runs with standards described in MATERIALS AND METHODS.

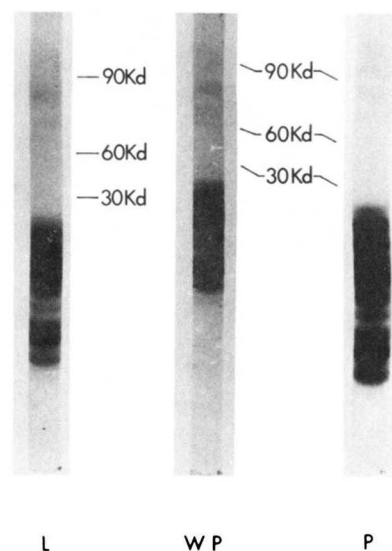


Fig. 2. Urea gel electrophoresis of cuticle proteins from larval, white puparial and amber puparial integument. Cuticles from the appropriate stages were extracted with 8 M urea-phosphate, pH 8.0 and resolved on 10% gels, containing 8 M urea. Bands were revealed with amido schwarz-B. Distance of migration of potential di- and trimers established with marker proteins. L, larvae; WP, white puparial; P, amber puparial stages.

TABLE I

Molecular weights, isoelectric points and yields of cuticle proteins purified by isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Focused proteins were purified on SDS-gels as described for Fig. 1. Molecular weight of each component was assessed by second runs on 10% SDS-gels (15). The relative yield was determined by amino acid recoveries following hydrolysis.

PROTEIN NO.	pI	MOLECULAR WEIGHT $10^{-3} M_r$	RELATIVE AMOUNT %
1St	4.51	23	1
2	4.63	20	1
3	4.68	17	8
4	4.73	21	3
5	4.80	16	5
6	4.88	22	7
7	4.90	21	6
8	4.97	22	13
9	5.03	24	3
10	5.08	21	11
11	5.13	21	2
12	5.18	21	9
13	5.23	21	2
14	5.33	20	3
15	5.38	19	7
16	5.50	17	1
17	5.53	17	1
18	5.75	17	3
19	5.88	18	7
20	5.90	19	5
21	5.96	16	1

TABLE II Amino acid composition of cuticle proteins. Values in residues per mole of protein rounded to the nearest whole number.

Protein Number	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	CB-1
Aspartic acid	25	23	30	29	39	35	30	35	30	31	29	20	36	38	19	17	22	25	22	6
Glutamic acid	30	32	46	34	48	47	42	48	41	38	36	31	36	42	38	32	29	30	39	12
Threonine	11	12	14	7	9	9	8	8	6	9	7	11	9	11	12	11	8	6	11	3
Serine	28	31	27	10	12	12	11	8	10	12	14	16	17	21	22	23	22	13	23	33
Proline	4	2	9	5	9	10	10	10	5	7	10	6	12	14	1	1	4	6	1	21
Glycine	38	31	34	22	29	25	22	27	25	28	27	26	49	57	36	28	25	17	35	24
Alanine	15	15	16	12	17	18	15	18	16	15	17	11	8	10	9	8	9	5	13	36
Valine	10	29	27	10	30	31	28	32	25	25	25	14	16	16	14	13	21	10	25	9
Isoleucine	6	6	7	5	6	6	8	11	9	10	12	7	7	6	5	6	8	10	8	0
Leucine	8	8	9	7	6	8	11	13	11	9	10	8	7	7	8	10	8	8	7	9
Tyrosine	7	7	6	5	10	11	9	10	7	7	7	5	4	2	3	5	6	6	7	12
Phenylalanine	3	3	3	3	2	5	8	9	5	5	5	4	2	1	4	2	4	3	n.d. <sup>a</sup>	
Histidine	29	12	11	2	21	17	16	19	11	14	23	48	9	12	8	5	11	17	10	6
Lysine	16	7	16	15	24	22	20	22	24	16	15	15	21	18	19	20	10	13	10	3
Arginine	1	1	1	0	0	0	1	2	1	1	2	1	0	0	0	2	1	2	1	3
$\beta$ -Alanine	1	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0
OH-lysine	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Acidic																				
Basic	1.2	2.7	2.7	3.5	1.9	2.1	1.9	1.9	2.0	2.2	1.6	1.3	2.8	2.7	2.1	1.8	2.3	1.7	2.6	
Polar																				
Non-Polar	2.2	1.2	1.5	1.9	1.9	1.6	1.4	1.4	1.5	1.4	1.3	2.3	2.1	2.0	2.2	1.8	1.4	2.0	1.5	
Amino Terminus	- gly	-	- gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

<sup>a</sup> n.d., not determined

TABLE III

Radioactivity of cuticle proteins from larvae administered [ $7\text{-}^{14}\text{C}$ ] dopamine. Borate-SDS-soluble proteins were prepared from prepupal wandering larvae, administered 0.8  $\mu\text{Ci}$  [ $7\text{-}^{14}\text{C}$ ] dopamine. The maggots were sacrificed after 4-6 hours and dissected and extracted as described under MATERIALS AND METHODS. Proteins were resolved by isoelectric focusing. After elution from the gels, individual bands were assayed for protein and radioactivity. The control values for non-specific binding were established by incubation of unresolved nonradioactive borate-soluble proteins with 1  $\mu\text{Ci}$  of [ $7\text{-}^{14}\text{C}$ ] dopamine followed by exchange dialysis and precipitation with ethyl alcohol prior to scintillation counting.

PROTEIN NUMBER	$^{14}\text{C}$	
	PER CENT OF TOTAL dpm <sup>a</sup>	dpm/mole
1	1.2	34
2	1.0	46
3	4.8	34
4	1.8	38
5	3.0	34
6	5.5	51
7	1.8	56
8	8.4	44
9	2.8	62
10	6.9	58
11	1.3	48
12	7.5	72
13	2.2	51
14	2.4	40
15	3.7	41
16	1.6	30
17	1.2	29
18	1.8	37
19	3.4	29
20	3.6	38
CB-I <sup>b</sup>	21.1	79
Control		
$^{12}\text{C}$ Proteins 1-20+ [ $7\text{-}^{14}\text{C}$ ] dopamine	-	0.8

<sup>a</sup>Cuticle only.

<sup>b</sup>Polypeptide only.

TABLE IV

Distribution and specific activity of cuticle proteins from three life stages administered [ $7\text{-}^{14}\text{C}$ ] Dopamine as postfeeding larvae.

Synchronized postfeeding larvae received 0.8  $\mu\text{Ci}$  [ $7\text{-}^{14}\text{C}$ ] dopamine and 0.3  $\mu\text{Ci}$  L-[4- $^3\text{H}$ ] leucine in 1  $\mu\text{l}$  by injection. Individuals retaining the maggot conformation sacrificed after 4-10 hours. A second group pupariated on dry shavings and the larval, puparial and adult integuments were extracted as described in METHODS AND MATERIALS. Each life stage represents 200 animals.

	EXTRACTION MEDIA									
	Recovery <sup>ab</sup>	Borate-SDS	Urea	NaOH	Insoluble					
	%	%	cpm/mg	%	cpm/mg	%	cpm/mg	%	cpm/mg	
Larval	3.2	68	2,600	6	3,200	3	2,400	18	5,200	
Puparial <sup>d</sup>	7.7	22	3,800	8	4,100	10	6,600	61	13,600	
Adult <sup>e</sup>	18.5	4	---	4	---	9	116,000	83	191,000	

<sup>a</sup> $^3\text{H}$  recoveries from larval and puparial cuticle did not exceed 95 dpm/mg.

<sup>b</sup>Per cent recovered from cuticle of total [ $^{14}\text{C}$ ] administered.

<sup>c</sup>Per cent of [ $^{14}\text{C}$ ] in cuticle recovered in each extract or residue.

<sup>d</sup>Amber colored.

<sup>e</sup>Adult abdominal cuticle, thorax and wings.

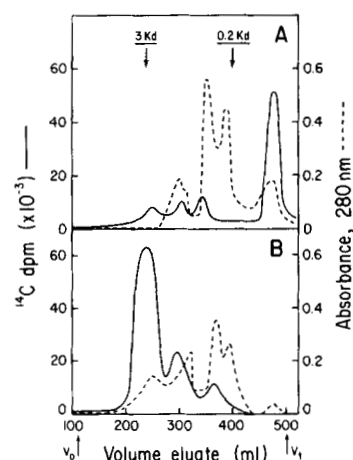


Fig. 3. Bio-Gel chromatography of proteolytic digests of cuticle from larvae and puparia receiving [ $7\text{-}^{14}\text{C}$ ] dopamine prior to pupariation. Borate extracts of the two integuments were digested serially with *S. aureus* V8 protease, subtilisin, Pronase, trypsin, carboxypeptidase A and pepsin at substrate: enzyme ratio of 50. After dialysis, the diffusate was chromatographed at 55°C on a column, 50 X 3 cm, with 0.1 M acetic acid for development. Polyaromatics and phenols are retarded under these conditions (20). Fractions of 5 ml were scanned at 280 nm and evaporated for determination of radioactivity. A, larval cuticle; B, amber puparial cuticle.