DEVELOPMENTAL BIOLOGY 86, 456-470 (1981)

The Cuticle of *Caenorhabditis elegans*

II. Stage-Specific Changes in Ultrastructure and Protein Composition during Postembryonic Development

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Received February 6, 1981; accepted in revised form April 13, 1981

The cuticle of the free-living nematode *Caenorhabditis elegans* is a proteinaceous extracellular structure that is replaced at each of four postembryonic molts by the underlying hypodermis. The cuticles of the adult and three juvenile stages (L1, Dauer larva, L4) have been compared ultrastructurally and biochemically. Each cuticle has an annulated surface and comprises two main layers, an inner basal layer and an outer cortical layer. The adult cuticle has an additional clear layer which separates the basal and cortical layers and is traversed by regularly arranged columns of electron-dense material. The fine structure of the cortical layer is similar in cuticles from different stages while that of the basal layer is stage specific. Purified cuticles were obtained by sonication and treatment with sodium dodecyl sulfate (SDS) and their component proteins solubilized with a sulfhydryl reducing agent. The degree of cuticle solubility is stage specific and the insoluble structures for each cuticle were localized by electron microscopy. Analysis of ^{35}S-labeled soluble cuticle proteins by SDS-polyacrylamide gel electrophoresis yields unique banding patterns for each stage. Most proteins are of high molecular weight (100-200 K) and are restricted to particular stages. Sixteen of the nineteen major proteins characterized are specifically degraded by bacterial collagenase. The results indicate that the different molts are not reiterative, but require the integration of both unique and shared gene functions. The potential use of stage-specific cuticle differences to identify and characterize regulatory genes controlling cuticle-type switching during development is discussed.

INTRODUCTION

Cuticle formation in the free-living nematode, *Caenorhabditis elegans*, shows promise for study of the genetic control of a complex developmental process (Cox *et al.*, 1980). The cuticle is a proteinaceous extracellular structure that encloses the animal. It is synthesized and secreted by an underlying layer of hypodermal tissue which, for the most part, consists of a single large syncytium that extends throughout the length of the animal (White, 1974). The entire cuticle is shed four times during the animal's 3-day development and a new cuticle is formed *de novo* at each molt (Singh and Sulston, 1978; Cox *et al.*, 1981a).

In a previous report we described the purification of intact cuticles from *C. elegans* adults and characterized the morphological and biochemical features of these cuticles (Cox *et al.*, 1981b). This report extends these studies to include three of the juvenile stages of *C. elegans*, the L1, dauer larva, and L4. We find that the cuticles of these stages exhibit remarkable differences in internal architecture which are reflected in the electrophoretic banding patterns of their component proteins. The results indicate that the different molts are not reiterative, but require the integration of both unique and shared gene functions. The implications of our findings for genetic studies of cuticle formation are discussed.

MATERIALS AND METHODS

Nematode Strains and Culture Methods

N2 is the wild-type strain of *C. elegans* var. Bristol and was obtained from Sydney Brenner (1974). fer-1(hclts)I and dae-2(e1370ts)III are temperature-sensitive ethyl methanesulfonate-derived mutants of N2 and have been described (Ward and Miwa, 1978; Riddle, 1977). At 25°C, daf-2 animals form dauer larvae constitutively (i.e., in the presence of food). At this temperature, fer-1 animals produce defective sperm and hermaphrodites are consequently self-sterile. Both mutants grow normally when reared at 16°C and daf-2 dauer larvae formed at 25°C molt into L4 juveniles when shifted to 16°C. Neither fer-1 nor daf-2 mutations alter the structure of the cuticle as judged by electron microscopy and we assume that they do not affect the

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0012-1606/81/120456-15$02.00/0

proteins of the cuticle as well. This assumption has been verified for fer-1 L4's and adults.

NG media, 8P media, NP media, and M9 buffer have been described (Brenner, 1974; Schachat *et al.*, 1977; Schachat *et al*; 1978). Unless noted otherwise, nematodes were grown on 100-mm agar plates using *Escherichia coli* strain OP50 as a food source.

Dauer larvae were obtained using egg-white plates (Cox, *et al.*, 1981b) and stored at 16°C in M9 buffer until use. daf-2 dauer larvae were also obtained by shifting heavily populated NG agar plates from 16 to 25°C and leaving them at this temperature for several days. Dauer larvae were purified from other stages by incubation with 1% sodium dodecyl sulfate (SDS) and centrifugation through 15% Ficoll as described by Cox, *et al.*, (1981b).

^{35}S-Labeling Procedures

^{35}S-Labeled bacteria were prepared by growing *E. coli* strain NA22 for 12 to 24 hr at 37°C in 30 to 50 ml of low-sulfate medium (Bretscher and Smith, 1972) supplemented with 0.2 to 0.5 mCi of $H_2{}^{35}SO_4$ (New England Nuclear, carrier-free). After harvesting, radioactive bacteria were suspended in M9 buffer (usually 2 ml of M9 buffer per 50 ml of bacterial culture) and spread onto 100-mm NP agar plates (0.5 ml per plate). These plates were allowed to dry and either seeded directly with nematodes or stored for up to 1 week at 4°C.

Labeled L4's were prepared by plating dauer larvae (N2 or fer-1) onto ^{35}S-*E. coli* plates (2 × 104 animals per plate) and allowing them to develop for 15 hr at 25°C, which is several hours after the completion of the dauer to L4 molt and a few hours before the onset of adult cuticle synthesis (Cox *et al.*, 1981a).

For labeling adult nematodes, fer-1 L4 juveniles grown from dauer larvae on 8P plates (14–15 hr at 25°C) were plated onto ^{35}S-*E. coli* plates (5–10 × 10^3 animals per plate) and allowed to develop until they were mature adults (an additional 14–16 hr at 25°C).

Radioactive dauer larvae were prepared by plating newly hatched daf-2 L1's onto ^{35}S-*E. coli* plates (3–5 × 10^4 animals per plate) and incubating the plates for 2 days at 25°C. Dauer larvae were isolated as described above.

For labeling L1's, young N2 adults were placed on ^{35}S-*E. coli* plates (1–5 × 10^3 animals per plate) for 8–12 hr, transferred to nonradioactive NG plates to lay eggs for 12–16 hr, and discarded. Newly hatched radioactive L1's were collected from these plates at 6-hr intervals. In one experiment, eggs were isolated by digesting ^{35}S-labeled adults with 1% NaOCl in 0.5 *M* NaOH for 7 min (R. Hecht, University of Houston, personal communication). Eggs are not affected by this treatment and were plated onto NG agar plates after several rinses with M9 buffer.

Cuticle Isolation Procedures

Cuticles were purified by sonicating nematodes in 10 m*M* Tris-HCl (pH 7.4), 1 m*M* EDTA, 1 m*M* phenylmethanesulfonyl fluoride and incubating the crude cuticle pieces in ST buffer (1% SDS, 0.125 *M* Tris-HCl (pH 6.8)) for 2 min at 100°C as described by Cox, *et al.*, (1981b). Sonication times used to disrupt the animals were: adults, 3 min; L4's, 1.8 min; dauer larvae, 4.5 min; and L1's, 1.2 min. Soluble cuticle proteins were obtained by heating purified cuticles in 0.2–0.5 ml of ST buffer, 5% β-mercaptoethanol (BME) for 2 min at 100°C and agitating gently for several hours at room temperature. BME-insoluble cuticle material was washed three times with 1 ml of ST buffer, 5% BME and either lyophilized, after several rinses with distilled water, or solubilized with Protosol (New England Nuclear) for scintillation counting. In radiolabeling experiments, aliquots were removed at various stages in the isolation procedure to monitor recovery of ^{35}S-labeled protein. Trichloroacetic acid (TCA)-soluble and insoluble radioactivity was determined as described (Cox *et al.*, 1981a). Protein samples were stored at −20°C until use.

Using the labeling procedures described above, yields of radioactive BME-soluble cuticle proteins were: adults (1–3 × 10^5 cpm/10^3 nematodes); L4's (3–8 × 10^4 cpm/10^3 nematodes); dauer larvae (2–4 × 10^4 cpm/10^3 nematodes); and L1's (0.8–1.5 × 10^3 cpm/10^3 nematodes).

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli and Favre (1973) with only minor modifications. Each gel lane contained 1–5 × 10^4 cpm. Gels were fixed overnight in 50% methanol, 10% acetic acid and treated either with PPO (2,5-diphenyloxazole) in dimethyl sulfoxide (Bonner and Laskey, 1974) or with EN^{3}HANCE (New England Nuclear and Amersham). The gels were dried and exposed to preflashed Kodak XR-5 X-ray film (Laskey and Mills, 1975) for 1–20 days at −70°C. The following ^{14}C-labeled proteins (New England Nuclear) were used as molecular weight standards: myosin (212,000), phosphorylase b (92,500), bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (29,000), and cytochrome *c* (11,700).

Collagenase Digestions

Radioactive soluble cuticle proteins of each developmental stage were mixed with 40 μg nonradioactive

soluble adult cuticle protein in ST buffer, 5% BME, and the proteins were precipitated with 9 vol of ice-cold acetone (Weber *et al.*, 1972). The precipitates were washed once with 90% acetone, air-dried, and incubated with 20 units of bacterial collagenase (Advanced Biofactures Corp. Form III) in 50 μl of 50 m*M* Tris-HCl (pH 7.4), 10 m*M* $CaCl_2$, 150 m*M* NaCl for 15 hr at 37°C. This collagenase is free of detectable nonspecific proteases (Miller and Udenfriend, 1970). In some experiments an additional 20 units of collagenase was added after the first 12 hr. Parallel control preparations were incubated with buffer minus enzyme. Digestions were terminated by dilution into electrophoresis sample buffer and heating for 2 min at 100°C. Samples were electrophoresed directly. After fixation, gels were stained with 0.1% Coomassie blue R-250 in 50% methanol, 10% acetic acid, destained in 5% methanol, 10% acetic acid, and prepared for fluorography.

Amino Acid Analyses

Lyophilized cuticle protein samples were hydrolyzed *in vacuo* for 24 hr at 110°C in 6 *N* HCl and processed on a Durrum D-500 amino acid analyzer. Cysteine was determined as cysteic acid in samples that were oxidized with performic acid (Hirs, 1956).

Microscopy

Nematodes and cuticle preparations were prepared for electron microscopy, as described by Cox *et al.* (1981b). A Wild M5 dissecting microscope was used to monitor the molting cycles (Cox *et al.*, 1981a).

IV. RESULTS

The postembryonic development of *C. elegans* is interrupted by four molts which divide the life cycle into five discrete stages termed L1, L2, L3, L4, and adult. During periods of adverse environmental conditions (e.g., starvation), L2 juveniles molt into a nonfeeding developmental alternative to the L3, called the dauer larva. Dauer larvae are more resistant to noxious chemicals than are the other stages and can survive for many times the normal life span (Cassada and Russell, 1975; Klass and Hirsh, 1976). Upon encountering suitable environmental conditions, the dauer molts into an L4 and thereby rejoins the regular developmental pathway.

In the following sections we describe the ultrastructure and protein composition of the L1, dauer, L4, and adult cuticles. L2 and L3 cuticles are not included because of difficulties encountered in obtaining large synchronous populations for biochemical purposes. Certain features of dauer and adult cuticles have been described previously (Cassada and Russell, 1975; Popham and Webster, 1978; Cox *et al.*, 1981b) but are included for comparison.

A. Cuticle Morphology

The surface morphology of the cuticle is similar at each developmental stage. The cuticles of all stages are

TABLE 1
RELATIVE SIZE CHARACTERISTICS OF JUVENILE AND ADULT CUTICLES

Developmental stage	Approximate length (μm)	Annula width[a] (μm)	Cuticle thickness range (μm)	Cuticle thickness to body diameter ratio[b]	Cuticle as percentage total body cross-sectional area[c]
Adult	1200	1.23 ± 0.24 1.72 ± 0.08	0.4–0.62	1:88	4.4
L4	800	1.12 ± 0.14 1.06 ± 0.07 0.96 ± 0.10	0.26–0.35	1:88	4.4
Dauer	500	1.45 ± 0.12 1.41 ± 0.19 1.58 ± 0.08	0.3–0.35	1:36	10.2
L1	250	1.64 ± 0.13 0.99 ± 0.04	0.1–0.15	1:88	4.4

[a] Data are the means ± 1 SD for 15–40 annulae of individual nematodes. Distance between annulae was measured from the base of the annular furrows.

[b] Determined from favorable transverse sections of two to three nematodes for each state. Cuticle thickness was subtracted from body diameter measurements.

[c] Determined using data in previous column and assuming that the cross section of a nematode is a circle.

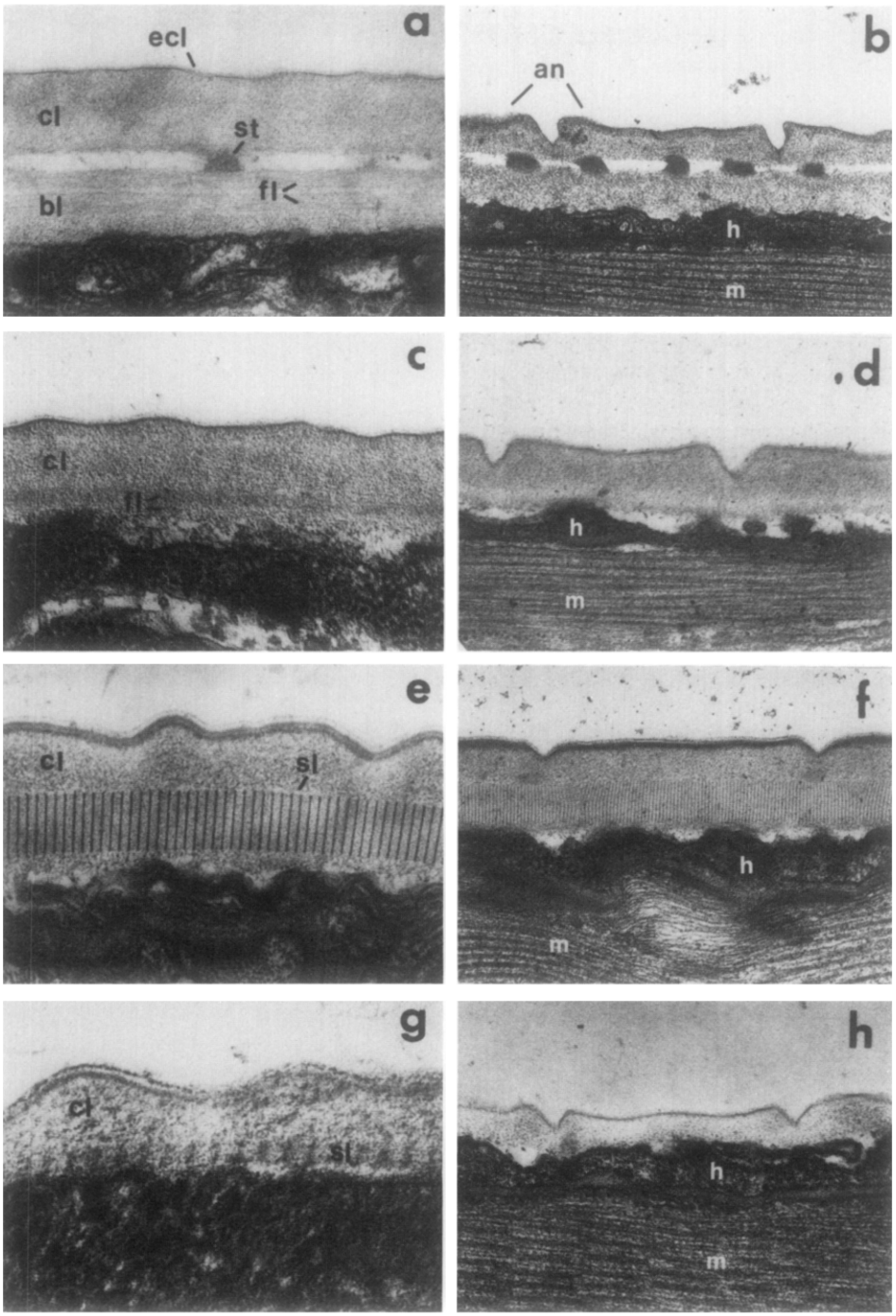

FIG. 1. Transmission electron micrographs of juvenile and adult cuticles. Transverse (left-hand column) and longitudinal (right-hand column) sections are shown as opposing pairs. Adult cuticle: (a) ×50,000 and (b) ×27,000; L4 cuticle: (c) ×70,000 and (d) ×34,000; dauer cuticle: (e) ×67,000 and (f) ×38,000; L1 cuticle: (g) ×170,000 and (h) ×70,000. Cuticle structures indicated are: an, annulae; bl, basal layer; cl, cortical layer; ecl, external cortical layer; fl, fiber layer; sl, striated layer; st, struts. Hypodermal (h) and muscle (m) cells are also indicated.

relieved by regularly spaced circumferential indentations termed annulae. The distance between annulae is similar in each stage and averages between 1 and 1.5 μm (Table 1). The variations in annulae width observed between individuals of different stages were similar to those observed between individuals of the same stage and therefore are probably not significant.

The only other major distinctive surface features of the cuticle are alae, which are longitudinal thickenings of the cuticle that run along the lateral sides of the

animal. Alae are present in L1, dauer, and adult cuticles, but not L4 cuticles (nor L2 or L3 cuticles; Singh and Sulston, 1978). The form of the alae is stage specific as is described below. When present, alae interrupt the otherwise continuous paths of the annulae around the worm.

As the animal matures, the cuticle formed at each successive stage is thicker (Table 1). The increase in cuticle thickness is proportional to the increase in body diameter during this time. In the L1, L4, and adult, the ratio of cuticle thickness to body diameter averages about 1:88 (Table 1). Dauer larvae, however, have a cuticle thickness to body diameter ratio of about 1:36, more than double that of the other stages. The reason for this appears twofold. First, dauer larvae are unusually thin (comparable in diameter to L1's) and possess a length to diameter ratio nearly double that of other stages (Cassada and Russell, 1975). Second, the dauer cuticle appears to be especially thick as it is equal to or greater in thickness than the L4 cuticle. No comparable measurements have been made for L3 juvenile cuticles.

The internal architectures of L1, dauer, L4, and adult cuticles are quite distinct. Transmission electron micrographs of each cuticle are presented in Fig. 1 and a description of each is given below. Nematode cuticles are typically divided into three regions: an inner (basal) zone, a clear (median) zone that is not always present, and an outer (cortical) zone (Bird, 1971). We have continued this nomenclature system in describing the different *C. elegans* cuticles.

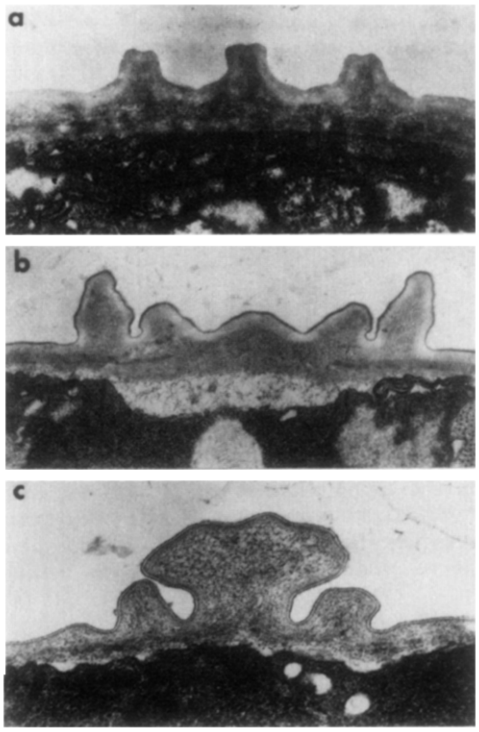

FIG. 2. Transmission electron micrographs of lateral alae (a) adult cuticle, ×25,000 (b) dauer cuticle, ×27,000; (c) L1 cuticle, ×75,000.

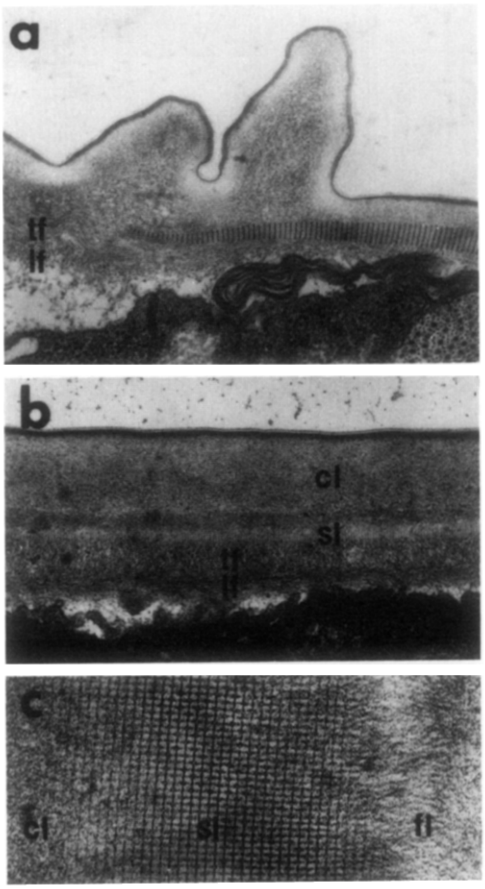

FIG. 3. Transmission electron micrographs of special features of the dauer cuticle. (a) The striated layer (sl) tapers at the lateral ridges and is replaced by tranverse (tf) and longitudinal (lf) fibers (×33,000). (b) Longitudinal section through the edge of the lateral alae which shows the underlying longitudinal fibers. The transverse fibers appear amorphous at this angle (×29,000). (c) Low-angle longitudinal section through the main body cuticle which shows the rectangular array of interwoven laminae that give rise to the striations seen in transverse and longitudinal sections. The cortical layer (cl) and loosely organized fibrillar material (fl) underlying the striated layer are indicated (×70,000).

1. The adult cuticle. The basal and cortical layers of the adult cuticle are clearly separated by an electron-transparent median layer that is probably fluid filled. Small, dense columns of material (termed "struts") traverse the median layer and are the only attachment points between the basal and cortical layers. Struts are organized into transverse rows on either side of the annular furrows and are absent beneath the alae. The cortical layer consists of a thin, electron-dense external cortical layer and a less dense internal cortical zone. The basal layer comprises a loosely organized fibrillar

layer adjoining the hypodermis and two layers of highly organized fibers that spiral around the animal in opposite directions, each at an angle of 60–70° relative to the worm's long axis. The adult alae are comprised of three ridges of about equal size (Fig. 2a). The surface features of the cuticle, the annulae, and alae, represent variations in the morphology of the cortical layer. The morphology of the basal layer, in contrast, appears uniform throughout the worm.

2. The L4 cuticle. The L4 cuticle, like the other juvenile cuticles, is organized as a single layer; a clear median layer separating the basal and cortical layers is absent. The external and internal cortical layers appear similar to the corresponding structures of the adult cuticle. The basal layer of the L4 cuticle contains two fibrillar layers that differ in their orientation. These fiber layers may be organized in a spiraling manner similar to those in the adult cuticle; however, this has not been determined. Alae are not present in the L4 cuticle and the structure of the cuticle at the lateral ridges is similar to that seen elsewhere in the worm.

3. The dauer cuticle. The most striking feature of the dauer cuticle is the striated zone present in the basal layer. Striations are observed in both transverse and longitudinal sections and derive from two sets of interwoven laminae, one of which is oriented longitudinally and the other circumferentially. The rectangular array of these laminae can be seen in glancing sections of dauer cuticles (Fig. 3c). The striated zone is separated from the hypodermis by a layer of loosely organized fibrillar material. The cortical layer appears similar to that of the other stages except that the external cortical layer is thicker. The dauer cuticle has broad five-pronged alae (Fig. 2b). Beneath the alae the striated zone tapers and is replaced by a layer of transverse fibers which overlay a layer of longitudinal fibers (Figs. 3a and b).

4. The L1 cuticle. In transverse sections osmiophilic striations are also seen in the basal layer of the L1 cuticle (Fig. 1g). These striations are shorter and less distinct than the striations seen in the dauer cuticle, although they are of the same periodicity (ca. 18 nm). No intermediate layer separating the striated layer from the hypodermis is present in L1 cuticles. In longitudinal sections, the basal layer appears fibrillar, not striated, and therefore may be organized in a manner different from the striated layer of the dauer cuticle. The fibrillar appearance of this layer may be an artifact, however, because L1's never fixed as well as the other stages. The structure of the cortical layer is similar to that of the other stages. The L1 alae comprise three ridges: the central ridge is much larger than the other two and is of variable morphology. In some sections the central ridge appeared mushroom-shaped (Fig. 2c), while in other sections (often in the same animal) it appeared V-shaped due to indentation of the top central portion of the ridge. Beneath the alae, the striated layer merges with a layer of dense transverse fibers which overlay a layer of more loosely organized fibrillar material.

B. Purification of Cuticles and Localization of Insoluble Structures

Cuticles were purified by sonication and treatment with SDS as described under Materials and Methods. Cellular material such as muscle bundles and basement membranes were never observed in electron micrographs of these cuticle preparations. Purified dauer (Figs. 4e and 5c) and L4 (Fig. 4c) cuticles have a morphology virtually indistinguishable from that of *in situ* cuticles. Adult cuticles (Figs. 4a and 5a) are also well preserved by these treatments except that the two fiber layers appear more disorganized than in unextracted cuticles and the alae typically have a buckled appearance, possibly due to contraction of the basal layer. Purified L1 cuticles (Figs. 4g and 5e) appear convoluted due to circumferential contraction of the basal layer and the alae appear broad and amorphous. On the whole, though, purified L1 cuticles appear largely intact and seem to have suffered no obvious loss of structural detail.

Cuticles of all stages are partially solubilized by sulfhydryl reducing agents such as BME. Electron micrographs of BME-treated adult cuticles (Figs. 4b and 5b) indicate that the entire basal layer, struts, and most of the internal cortical layer have been removed. Only the external cortical layer remains intact and is associated with a small amount of internal cortical layer material. The entire basal layer and most of the internal cortical layer is also removed from L4 cuticles (Fig. 4d). More of the internal cortical layer remains insoluble in L4 cuticles than in adult cuticles and this material often appears as partially extracted clumps that have pulled away from the external cortical layer. The gross morphology of dauer cuticles is only mildly affected by BME (Figs. 4f and 5d). The only structure extensively solubilized is the loosely organized fibrillar material underlying the striated zone. Portions of the internal cortical layer, the transverse and longitudinal fibers underlying the alae, and the material comprising the interstices of the lattice structure that gives rise to the striated zone, are also removed. Dauer cuticles shrink when treated with BME due to contraction of the striated layer. Contraction of this layer causes the attached cortical layer material to buckle at fairly regular intervals. The L1 cuticle structures that are BME-insoluble are the external cortical layer and portions of the striated layer; the internal cortical layer is

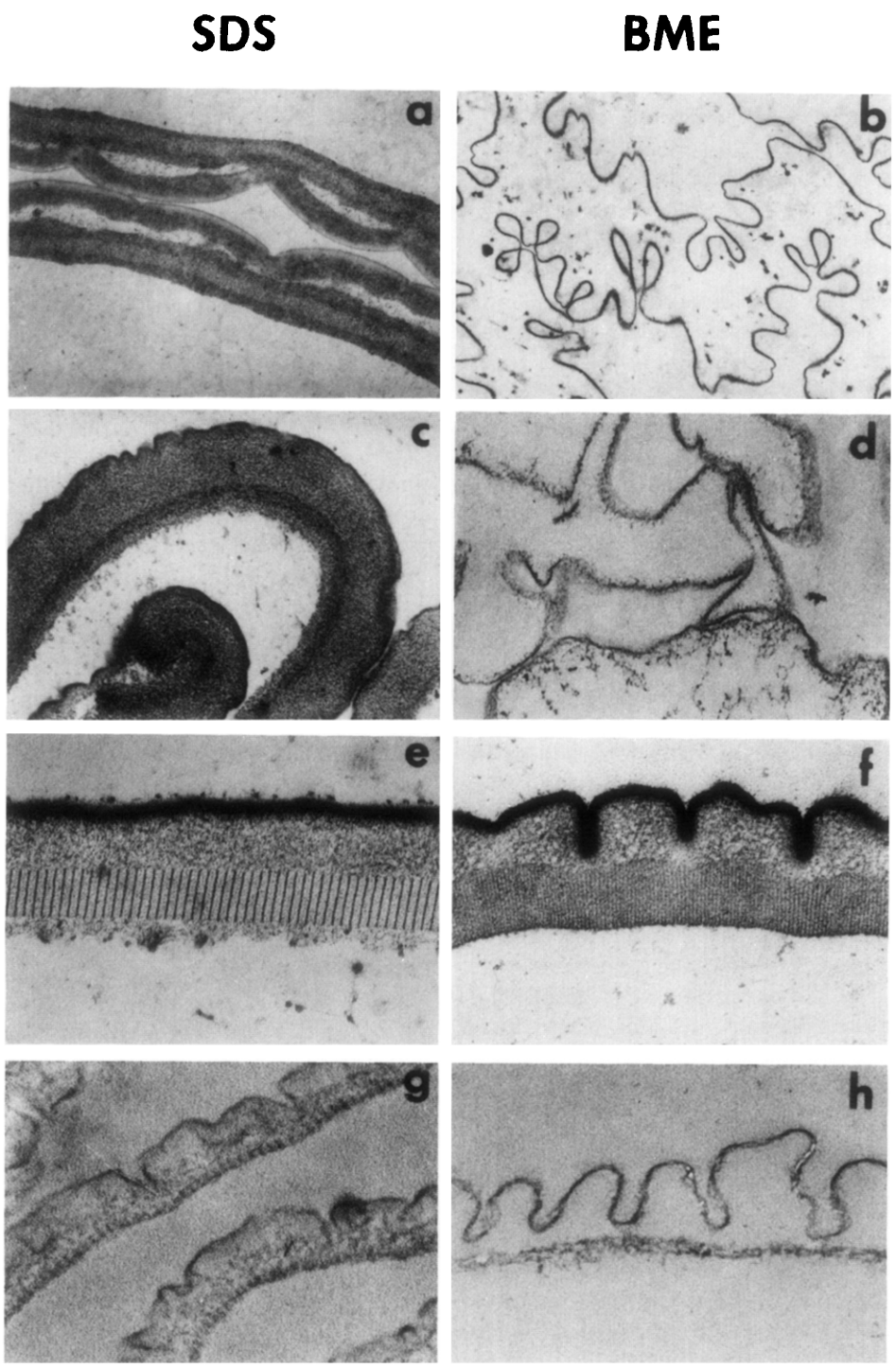

FIG. 4. Transmission electron micrographs of purified cuticles before and after treatment with β-mercaptoethanol. SDS-purified (left-hand column) and BME-treated (right-hand column) cuticles are shown as opposing pairs at identical magnifications. Adult cuticle: (a) and (b), ×10,000; L4 cuticle: (c) and (d), ×33,000; dauer cuticle: (e) and (f), ×74,000; L1 cuticle: (g) and (h), ×110,000.

largely removed (Figs. 4h and 5f). The striated layer appears fibrillar and loosely organized, suggesting that some structural components have been extracted. The external cortical layer appears highly convoluted and seems to be attached at specific points to the remains of the striated layer.

A summary of these observations is presented in Fig. 6.

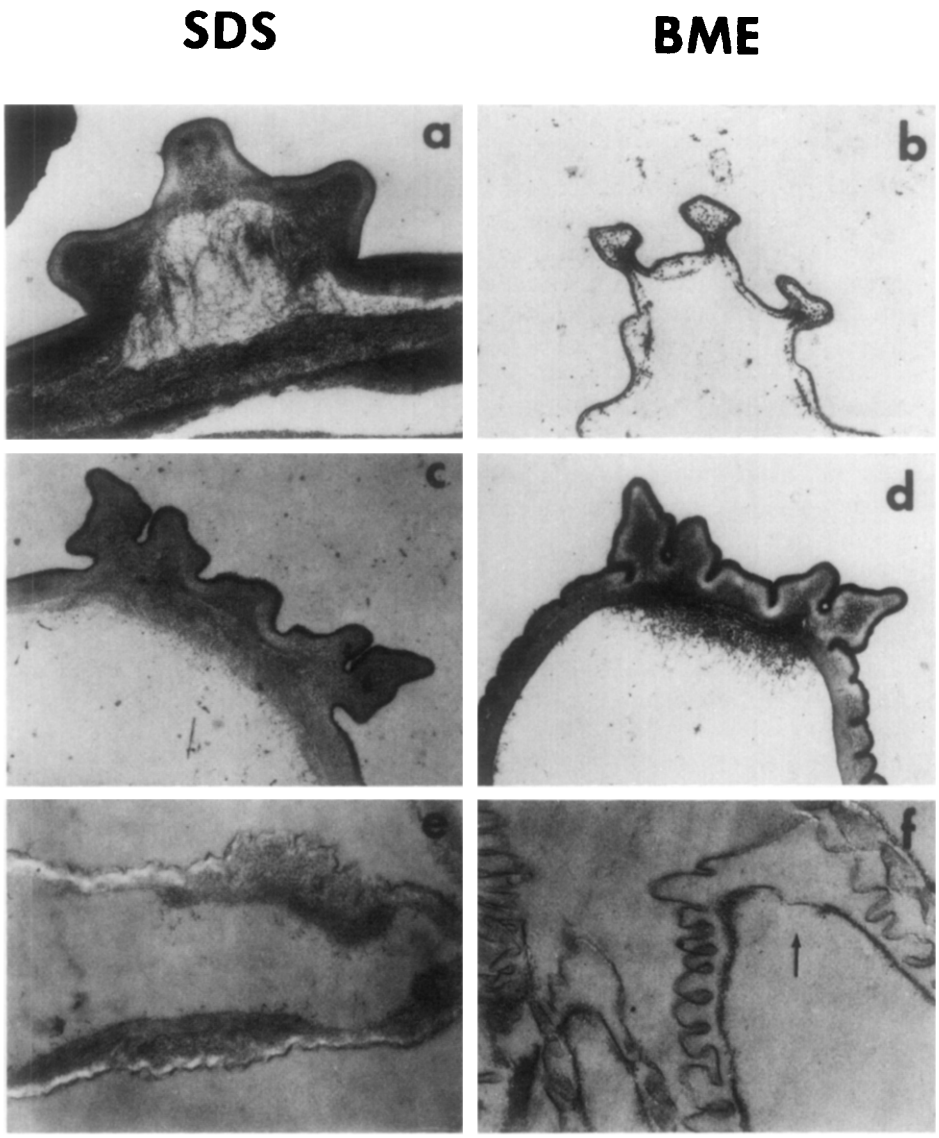

FIG. 5. Transmission electron micrographs of lateral alae before and after treatment with β-mercaptoethanol. SDS-purified (left-hand column) and BME-treated (right-hand column) alae are shown as opposing pairs at identical magnifications. Adult alae: (a) and (b), ×17,000; dauer alae: (c) and (d), ×17,000; L1 alae: (e) and (f), ×27,000; L1 alae are difficult to discern after treatment with BME and are indicated with an arrow in (f).

C. Synthesis of Cuticle Proteins during the Molts

The strategies for specifically labeling L1, dauer, L4, and adult cuticles with ^{35}S are described under Materials and Methods. In labeling L1, L4, and adult cuticles, animals were given essentially single-stage (molt) pulses of radioactivity; therefore, the amount of radioactivity recovered in these cuticles vs the rest of the animal provides an estimate of the proportion of total protein synthesis during these stages (molts) that is devoted to cuticle synthesis (assuming equal turnover rates for all proteins). As indicated in Table 2, purified cuticles account for 6–9% of the total ^{35}S-labeled protein recovered from these stages. In contrast, cuticle material represents 23% of the total labeled protein recovered from dauer larvae even though these animals were labeled continuously from the L1 stage and thus underwent two molts. For each stage examined, the percentage of total labeled protein recovered in the cuticle is about 1.5–2 times that expected from consideration of the cross-sectional area of the animal occupied by the cuticle (Table 1).

Table 2 also gives the percentage of ^{35}S-labeled cuticle material for each stage that is solubilized by BME. In general, these values correlate well with the electron microscopy results. The data indicate that the majority of the ^{35}S-labeled protein in L4 and adult cuticles, 70 and 75%, respectively, is BME soluble, whereas only

46% of the protein in the L1 cuticle and 26% of the protein in the dauer cuticle is removed by BME.

D. Characterization of BME-Soluble Cuticle Proteins by SDS-PAGE and Collagenase Sensitivity

^{35}S-Labeled BME-soluble cuticle proteins of each stage were separated by SDS-PAGE and visualized by fluorography. A 7½% gel is shown in Fig. 7 and 5 and 10% gels are shown in Fig. 8. Several gel concentrations are necessary to resolve all cuticle proteins because a few proteins (e.g., cpI and cpJ, see Table 3) show increased mobilities in higher percentage gels and as a consequence comigrate with other proteins depending upon the gel concentration. Comparison of the protein banding patterns of the different stages reveals that each is unique. Each cuticle yields a limited number of major protein components in the 50–200K molecular weight range, many minor components of very high molecular weight (>200K) and a limited number of minor components of low molecular weight (<50K). Except for minor variations in the intensities of cpI and cpJ, the relative proportions of the different protein species was very reproducible. None of these proteins are extracted from the cuticle in the absence of BME (gels not shown).

Table 3 summarizes the proteins present in the cuticles of the different stages. In deriving this table we have limited our analysis to only the major components of each stage, and for the present, have assumed that cuticle proteins from different stages which comigrate on various percentage polyacrylamide gels, have similar band morphologies, and exhibit the same sensitivity to bacterial collagenase (see below) are identical; we realize that this assumption may have to be modified upon further experimentation.

Cuticle proteins fall into three general classes based on their developmental expression. The majority of cuticle proteins are unique to individual stages. A few proteins appear to be present in the cuticles of more than one, but not all stages (e.g., cpE in the L4 and adult; cpL in the L1 and L4). Only cpH and probably cpI and cpJ appear to be present in the cuticles of all stages (the latter two proteins are present in only very minor quantities in the adult cuticle).

Previous studies (Cox *et al.*, 1981b) established that the eight major proteins in the adult cuticle (cpA through cpH) are extensively degraded by bacterial collagenase. We have investigated the effect of this enzyme on the juvenile cuticle proteins. For these experiments ^{35}S-labeled BME-soluble cuticle proteins of each stage were mixed with unlabeled adult cuticle protein, precipitated with 90% acetone, incubated with collagenase, and the digestion products were analyzed by SDS-PAGE. Adult cuticle protein was included in these experiments so that the completeness of the digestions could be monitored by Coomassie staining prior to fluorography. All digestions were complete by this criterion. As shown in Fig. 8 and summarized in Table 3, all but three of the major juvenile cuticle proteins are specifically digested by this enzyme. Two of the collagenase-resistant proteins, cpP and cpQ, are unique to the L1, whereas the third, cpI, is present in variable amounts in all stages, although most prominently in the L1. Most of the minor, very high-molecular-weight bands (>200K) for each stage (except the dauer) are absent after treatment with collagenase. Most of the low-molecular-weight (<50K) minor proteins, particularly those in L1, are refractory to this enzyme. Three new bands (indicated by asterisks in Fig. 8) appear among the collagenase-resistant products of each set of proteins. These proteins are not seen in control samples and probably derive from some of the very high-molecular-weight proteins (>200K). These three proteins are very prominent in the L1 and show increased mobilities in higher-percentage polyacrylamide gels.

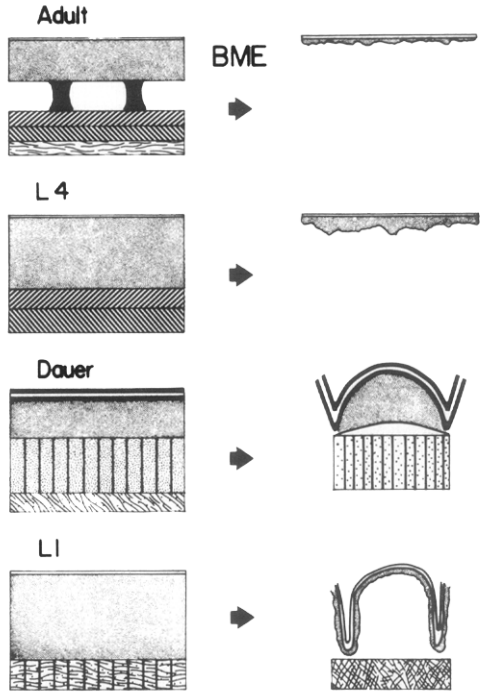

FIG. 6. Diagrammatic sketch summarizing cuticle structures solubilized by β-mercaptoethanol.

E. Amino Acid Compositions of Cuticle Proteins

Amino acid compositions of BME-soluble and insoluble cuticle proteins of dauer larvae and L4's were determined and are compared to the compositions of the corresponding adult proteins in Table 4. L1 proteins were not analyzed because of insufficient material. The soluble cuticle proteins of all stages have amino acid profiles typical of collagens; these proteins are rich in glycine, alanine, proline, hydroxyproline, and the acidic

TABLE 2
RECOVERY OF ^{35}S-LABELED PROTEIN AT VARIOUS STAGES IN THE PURIFICATION OF CUTICLES[a]

Developmental stage	Percentage total ^{35}S-labeled protein recovered			Percentage total cuticle radioactivity	
	Sonication supernatant[b]	SDS extract of cuticles	Cuticle	BME-soluble proteins	BME-insoluble proteins
Adult	83 (82-84)	8.2 (7.0-9.4)	8.7 (8.4-8.9)	75 (71-79)	25 (21-29)
L4	81 (78-86)	11 (8.3-14)	7.7 (6.1-8.4)	70 (67-73)	30 (70-77)
Dauer	56 (51-60)	21 (18-25)	23 (22-24)	26 (23-30)	74 (70-77)
L1	84 (77-89)	10 (6.9-16)	6.0 (4.6-7.5)	44 (42-47)	56 (53-58)

[a] Data are the means plus the range of values (parentheses) for four independent determinations for each developmental stage. Values above 10% have been rounded off to the nearest whole number.
[b] Determined as TCA-insoluble radioactivity.

amino acids, and poor in the basic and aromatic amino acids. As expected, these proteins contain significant amounts of cysteine. The soluble proteins of L4 and adult cuticles are nearly identical in composition; whereas those of the dauer cuticle show many distinct differences. Most significantly, the dauer proteins contain more glycine and cysteine and less hydroxyproline and alanine than the L4 and adult proteins. The ratio of hydroxyproline to proline residues is about 0.6 in the dauer proteins vs about 1 in the L4 and adult proteins. Dauer proteins contain two to three times as many hydrophobic residues as the other stages and also contain small quantities (0.1%) of hydroxylysine, which was not detected in L4 or adult proteins.

The BME-insoluble cuticle proteins of these stages have compositions similar to, but distinct from, each other and from the soluble proteins of each stage. The major compositional difference between the insoluble proteins of the different stages is in the levels of glycine and alanine residues, which are largely inversely proportional. The insoluble proteins of L4 and adult cuticles have more alanine and valine residues and fewer basic amino acids and hydroxylated proline residues than the soluble proteins of these stages. The insoluble dauer cuticle proteins are richer in glycine, alanine, and the imino acids, and poorer in the hydrophobic and basic amino acids than are the soluble dauer proteins. Hydroxylysine was not detected in the insoluble cuticle proteins of any stage; however, these proteins did contain small amounts (<1%) of two ninhydrin-positive peaks that eluted between phenylalanine and histidine. The identity of these peaks in not known.

DISCUSSION

Although the gross morphological and biochemical events of the different molts in the *C. elegans* life cycle appear similar (Singh and Sulston, 1978; Cox *et al.*, 1981a), our studies have shown that the cuticles of at least four stages differ substantially from one another in ultrastructure and protein composition. This finding indicates that the postembryonic development of the hypodermis entails not only a temporal pattern of discontinuous cellular activity (i.e., the molting cycles), but also a precise program of genetic switches which allow the production of distinctly different cuticles at different molts.

All nematodes exhibit a similar program of postembryonic development involving four molts. Stage-specific differences in cuticle architecture have been reported for selected stages of many nematode species, and are particularly common among the parasitic forms (for reviews see Chitwood and Chitwood, 1974; Bird, 1971). However, a systematic analysis of cuticle structure at each developmental stage has been performed with only one other nematode, the free-living *Panagrellus silusiae*. In this case no ultrastructural differences were observed and recent analysis of BME-soluble cuticle proteins revealed only quantitative variations between stages (Samoiloff and Pasternak, 1968; Leushner *et al.*, 1979). No comparative biochemical data exist for other nematodes like *C. elegans* that change cuticle structure during development; therefore we do not know how typical our findings will prove to be. Stage-specific differences in ultrastructure and protein electrophoretic banding patterns have been reported for certain insect cuticles (Roberts and Willis, 1980).

Some features of cuticle architecture are conserved among the various *C. elegans* stages. Each cuticle is organized into two main layers and each has an annulated surface. The structure of the cortical layer in each cuticle is similar. The surface features of the cuticle, the annulae, and alae are created through vari-

ations in the thickness and morphology of the cortical layer. Microscopy studies have suggested that the patterning of these features is largely the result of surface membrane activities of the underlying hypodermis (Lee, 1970; Singh and Sulston, 1978; our unpublished results). During deposition of the new cuticle, the hypodermal surface folds into regular arrays and appears to act as a template upon which the new annulae are formed. The close similarity in annulae width on all cuticles studied suggests that the same mechanism operates at each molt to generate these features. The lateral hypodermal seam cells are specifically responsible for the formation of the alae (Singh and Sulston, 1978). During the L4 to adult molt, the seam cell membrane forms three ridges from which the alae appear to be secreted. L1 and dauer alae, however, are secreted as broad bands by an enlarged seam and gain their characteristic shapes extracellularly through the action of as yet undetermined forces (Singh and Sulson, 1978; J. J. Sulston, personal communication). The broad amorphous appearance of L1 alae after treatment with SDS may be a reversion of these structures to their secreted form. The lack of a similar shape change in dauer alae may be a consequence of the high degree of crosslinking in this cuticle. Seam cells appear to be synthetically active at all molts (Sulston and Horvitz, 1977); therefore, at some molts (e.g., the dauer to L4 molt) these cells must follow the same synthetic and secretory program as the major hypodermal syncytium, whereas at other molts they apparently must follow distinct programs to generate alae. The greatest morphological differences between the various cuticles occur in the structure of their basal layers. Although each is unique, similarities between certain stages are evident. For example, the basal layers of both dauer and L1 cuticles contain radial striations that are replaced at the lateral ridges by transverse fibers while the basal layers of both L4 and adult cuticles are organized into layers of fibers.

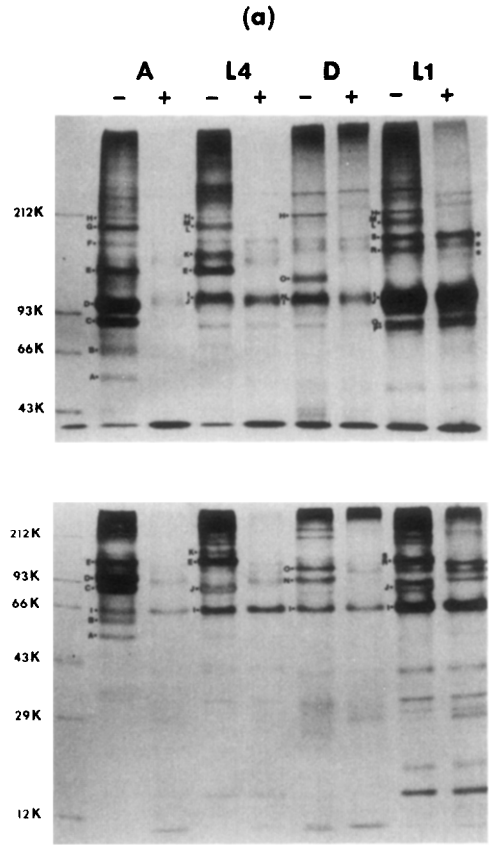

FIG. 8. Autofluorogram of control and collagenase-treated ^{35}S-labeled BME-soluble cuticle proteins separated by electrophoresis on 5% (a) and 10% (b) SDS-polyacrylamide gels. Samples containing 2.5 $\times 10^4$ cpm were mixed with 40 μg unlabeled adult cuticle protein, precipitated with acetone, and incubated with 20 units of collagenase (+) or with buffer only (−) for 12 hr at 37°C. An additional 20 units of collagenase or an equivalent volume of buffer was then added and the mixtures were incubated for an additional 3 hr at 37°C. After electrophoresis, gels were stained with Coomassie blue to verify digestion of the unlabeled adult protein, prepared for fluorography, and exposed to X-ray film for 8 days at −70°C. Labels are as indicated in the legend to Fig. 7.

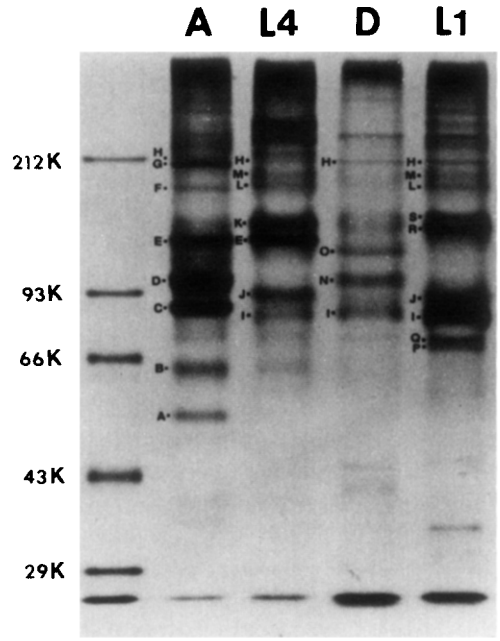

FIG. 7. Autofluorogram of ^{35}S-labeled BME-soluble cuticle proteins separated by electrophoresis on a 7½% SDS-polyacrylamide gel. Developmental stages are indicated above the gel lanes and the approximate molecular weights of protein standards are indicated in the far left column. Adult and dauer stages are abbreviated as A and D, respectively. Only major cuticle proteins are labeled. Each gel lane contains 2.5 $\times 10^4$ cpm and was exposed to X-ray film for 7 days at −70°C.

TABLE 3

APPROXIMATE MOLECULAR WEIGHTS, COLLAGENASE SENSITIVITY, AND DEVELOPMENTAL EXPRESSION OF *C. elegans* CUTICLE PROTEINS[a]

Class	Band	Approximate molecular weight[b]	Collagenase sensitivity[c]	Developmental stage			
				L1	Dauer	L4	Adult
I							
(Single stage)	cpA	53,000	Y				++
	cpB	67,000[d]	Y			(+)	++
	cpC	87,000	Y				+++
	cpD	101,000	Y				+++
	cpF	167,000	Y				+
	cpG	196,000	Y				++
	cpK	146,000	Y			+++	
	cpN	103,000	Y		+++		
	cpO	125,000	Y		++		
	cpP	77,000	N	+			
	cpQ	80,000	N	+			
	cpR	147,000	Y	++	(+)		
	cpS	159,000	Y	++	(+)		
II							
(Some stages)	cpE	129,000	Y			+++	++
	cpL	185,000[d]	Y	+		+	
	cpM	191,000[d]	Y	+		+	
III							
(All stages)	cpH	210,000	Y	+	+	+	+
	cpI	98,000[d]	N	+++	++	++	+
	cpJ	98,000[d]	Y	+++	+	++	+

[a] Proteins are grouped into classes based on their developmental expression and the intensity of each protein relative to other proteins within a stage is indicated. +++, major; ++, intermediate; +, minor. No sign indicates that the protein was not detectable. (+) indicates that a minor protein is present, but that a definite identification could not be made.

[b] Molecular weights given were determined on 5% SDS-polyacrylamide gels.

[c] Y, yes; No, no.

[d] These proteins exhibit significantly reduced molecular weights on higher percentage polyacrylamide gels.

The functioning of the cuticle as a hydrostatic skeleton (Harris and Crofton, 1957; Inglis, 1964) requires that it be anisometric. The cuticle must be capable of flexing longitudinally but not radially. This requirement may explain some of the similarities and differences seen in the various *C. elegans* cuticles. The basal layer is thought to be responsible for the radial strength of the cuticle whereas the cortical layer and annulae are designed to provide flexibility. As the animal grows, the internal hydrostatic pressure must increase and be counterbalanced by an increase in the radial strength of the cuticle. Inglis (1964) postulates that this requirement may have led to the evolution of molting and changes in internal cuticle morphology seen in many nematode species. This explanation is not wholly satisfactory, however, because some nematodes such as *P. silusiae* do not change cuticle structure at different molts but simply make a thicker version of the same cuticle. In this regard, our preliminary results suggest that the cuticles of the L2 and L3 stages of *C. elegans* are virtually identical to that of the L4 (M. Kusch and G. Cox, unpublished results). Many nematodes are also capable of considerable growth between molts and after the final molt (e.g., *Ascaris* adults increase in length nearly 30-fold without molting (Watson, 1965)). Thus, while growth is generally accompanied by an increase in cuticle thickness, it is not always accompanied by marked changes in cuticle structure. As an alternative explanation, we suggest that the cuticle changes seen in *C. elegans* may be an evolutionary vestige of a parasitic ancestry. Stage-specific differences in cuticle morphology are common among parasitic nematodes and in some cases are clearly of adaptive significance to particular environments or hosts (Bird, 1971; Chitwood and Chitwood, 1974). While *C. elegans* has been considered free living, at least one member of the genus and numerous members of the *Rhabditis* family are insect parasites (Poinar, 1972). Thus, it is possible that *C. elegans* is a secondarily derived free-living form and so retains developmental features characteristic of the ancestral parasitic forms.

The procedure used to purify cuticles does not sig-

TABLE 4
AMINO ACID COMPOSITION OF BME-SOLUBLE AND INSOLUBLE CUTICLE PROTEINS OF DAUER LARVAE, L4'S AND ADULTS[a]

Amino acid	Soluble cuticle proteins			Insoluble cuticle proteins		
	Adult[b]	L4	Dauer	Adult[b]	L4	Dauer
	Residues/1000 total amino acids					
Cysteine[c]	30	33	58	25	32	35
Hydroxyproline	120	105	54	75	67	81
Aspartic acid	80	98	77	59	51	66
Threonine	22	29	34	26	24	26
Serine	38	32	38	44	42	49
Glutamic acid[d]	100	95	87	110	99	60
Proline	113	104	92	134	134	127
Glycine	263	262	294	224	266	335
Alanine	112	133	75	195	165	142
Valine	19	20	34	34	30	13
Methionine	2.5	1.5	7.6	2.1	1.3	3.1
Isoleucine	10	13	22	9.2	15	9.9
Leucine	14	16	38	14	22	9.0
Tyrosine	5.2	4.3	10	7.5	6.6	3.4
Phenylalanine	9.6	9.5	18	8.8	11	5.0
Histidine	11	8.3	10	10	6.1	5.0
Hydroxylysine	0	0	1.2	0	0	0
Lysine	23	23	25	11	14	21
Arginine	30	16	25	14	14	11

[a] Averages of at least three hydrolysates of different cuticle preparations.
[b] Data from Cox, Kusch, and Edgar (manuscript submitted).
[c] Determined as cysteic acid.
[d] Includes the amidic forms.

nificantly alter cuticle morphology (except mildly the L1 cuticle) and therefore probably does not result in the loss of any major structural protein components. Both the electron microscopy and radiolabeling results indicate that the different cuticles vary markedly in the degree to which they are solubilized by disulfide reducing agents: L4 and adult cuticles are nearly completely solubilized whereas dauer cuticles are only mildly affected. The L1 cuticle is intermediate between these extremes. The cuticle material solubilized is generally restricted to particular structures. The external cortical layer was not solubilized in any cuticle. The internal cortical layer was nearly completely solubilized in L1, L4, and adult cuticles, but only minimally in dauer cuticles. This was true of the internal cortical layer material comprising the alae as well. The basal layer was completely solubilized in L4 and adult cuticles, but remained largely insoluble in L1 and dauer cuticles. The insolubility of the striated layer material in L1 and dauer cuticles supports the notion that these structures are homologous. The thick external cortical layer and high degree of covalent cross linking in the dauer cuticle correlates with the impermeability and presumed protective function of this cuticle (Bird, 1971; Cassada and Russell, 1975). The types of covalent bonds responsible for the insolubility of cuticle material remain to be determined.

The fact that the morphological variation in the different cuticles is reflected in the electrophoretic banding patterns of their component proteins suggests that specific proteins are used to form particular cuticle structure. If this were the case then we can begin to make inferences as to the locations of certain proteins within the cuticle. For example, on this notion we would predict that neither cpE nor cpH are strut-specific proteins because both proteins are present in stages other than the adult. Also, we would predict that none of the proteins analyzed derive from the external cortical layer. Because so much of dauer and L1 cuticles remain insoluble, it is possible that the apparent absence of a protein from these cuticles is due to solubility differences. The use of antibodies and analyses of mutants lacking specific cuticle proteins should aid in determining if this is the case. Our results seem to rule out the possibility that the different cuticles are comprised of similar sets of proteins that serve completely overlapping functions. If this were the case then only quantitative variations should have been observed in the electrophoretic banding patterns between stages.

We cannot say at this time whether the complexity

of the protein patterns of the different cuticles results from the existence of a large number of structurally unique proteins or as the result of a variety of post-translational modifications of a small set of proteins. Certain of the proteins have molecular weights and peptide maps (J. Politz, personal communication) which are consistent with the notion that they may be cross linked aggregates of lower molecular weight species (e.g., in the adult cpG may be a dimer of cpD). This is almost certainly the case for the very high-molecular-weight minor proteins (>220K) in each cuticle. Certain of the proteins (e.g., cpI and cpJ) also show increased mobilities in higher percentage polyacrylamide gels, suggesting that they may be glycosylated (Segrest and Jackson, 1972). The significant differences in amino acid composition between the dauer cuticle proteins and those of the L4 and adult (even when the whole cuticle is considered) provide further indication that these cuticles differ from one another biochemically, but again the differences could be due to the presence of unique proteins or to varying ratios of a similar set of proteins. Irrespective of the molecular origin of cuticle protein diversity, it is clear that the hypodermis has the genetic potential for generating a great variety of cuticle proteins and that this information is selectively expressed in series of overlapping patterns at different molts in the animal's life cycle.

Clostridial collagenase specifically cleaves the X-Gly bond in the sequence Pro-X-Gly-Y-Pro (where X and Y can be any amino acid) and therefore is a sensitive probe for collagen primary structure (Seifter and Harper, 1971). Sixteen of the nineteen major soluble cuticle proteins analyzed are extensively degraded by this enzyme and on this basis appear to be collagens. The amino acid composition of these proteins as a whole is also typical of collagens. The three major proteins not digested by this enzyme and the three minor proteins which appear after collagenase treatment would seem to be noncollagen proteins. *Clostridial* collagenase does not readily digest native collagens of certain annelid cuticles (Goldstein and Adams, 1970; Kimura and Tanzer, 1977); therefore, the nondigested proteins may represent special collagen forms which are protected in some way, perhaps through glycosylation, from the action of this enzyme.

The stage-specific differences in cuticle architecture and protein composition clearly indicate that the molts are not reiterative; rather each molt appears to integrate series of shared events (e.g., patterning of the annulae) with series of unique events (e.g., patterns of proteins produced). Thus, the products of some cuticle genes must function at all molts while others must function at only particular molts. This finding may account for the stage specificity in the phenotypic expression of many of the morphological mutants (e.g., blister and roller mutants) thought to play a role in cuticle formation (Brenner, 1974; Cox *et al.*, 1980). Two genes that appear to participate in controlling the normal program of cuticle switches during development have recently been described, *lin-4II* and *lin-14X* (Sulston and Horvitz, 1981; H. R. Horvitz, personal communication). Mutants defective for these genes were originally isolated with aberrations in postembryonic cell lineages, including hypodermal cell lineages: *lin-4(e912)* causes certain lineages to be reiterated while *lin-14(n925)* causes certain lineages to be "skipped." Further analyses have revealed that these mutants are also aberrant in certain cuticle transitions: *lin-4(e912)* possesses a juvenile cuticle as a sexually mature adult and *lin-14(n925)* possesses an adult cuticle as an L4 (Sulston and Horvitz, 1981; H. R. Horvitz, personal communication; our unpublished results). The precise effects of mutations in these genes on both cell lineage and cuticle patterns suggests that these genes play a role in regulating stage transitions during development. Since the structure and protein composition of the cuticle are sensitive assays of the developmental age of the hypodermis, it should be possible to exploit stage-specific cuticle differences (and cuticle mutants) to identify and characterize additional genes in the regulatory network that controls cuticle type switching during development.

We wish to thank Katherine Kanagaki at the University of California, Davis, for performing the amino acid analyses and our colleagues; Drs. Jerry Feldman, Meredith Kusch, Clifton Poodry, and Joan Politz, for their comments on the manuscript. This work was supported by Grant PCM 78-09439 from the National Science Foundation.

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