The Cuticle of Caenorhabditis elegans[1]

ROBERT S. EDGAR[2], GEORGE N. COX[3], MEREDITH KUSCH[2], AND JOAN C. POLITZ[2]

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The nematode cuticle i s among the most complex extracellular structures produced by a living organism. For the last few years the work in our laboratory has been devoted to the study of the cuticle of the free living nematode, *Caenorhabditis elegans*. Studies so far largely have been of a descriptive nature, involving attempts to characterize the morphology and composition of the cuticle and to isolate and study mutants altered in genes that control and regulate cuticle formation. Our long-term interest is to understand the genetic control and regulation of complex processes such as cuticle formation.

ADULT MORPHOLOGY

The external and internal morphology of the adult cuticle of *C. elegans* differs little from that of the sibling species, *C. briggsae*, examined by Zuckerman et al. (17). On the

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[2]Department of Biology, Thimann Laboratories, University of California, Santa Cruz, CA 95064.

[3]Department of Molecular, Cellular, and Developmental Biology; University of Colorado, Boulder, CO 80309.

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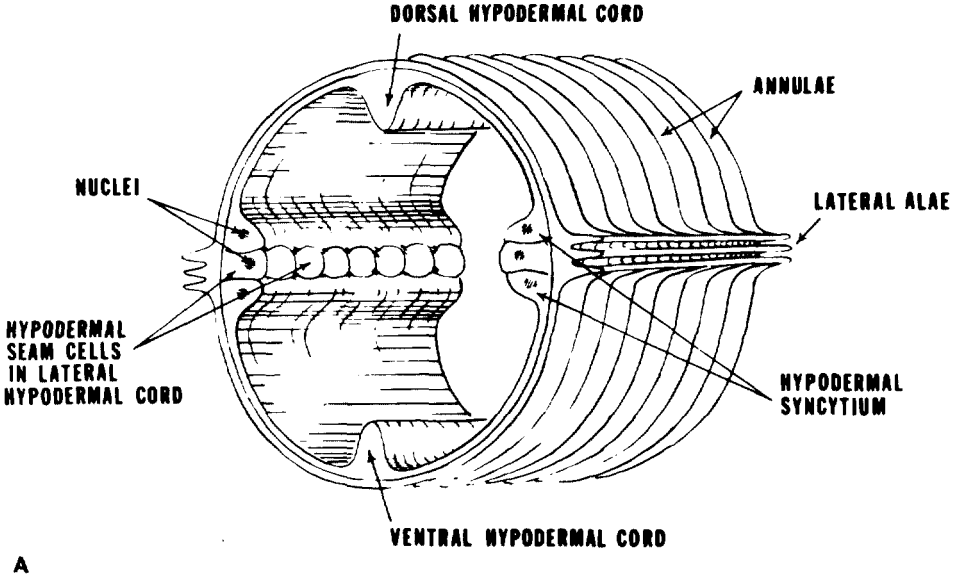
basis of our studies (3,5), the external cuticle surface revealed in SEM has more than 1,000 annulae 1.0 μm wide separated by furrows running circumferentially around the animal. These annulae are interrupted at the lateral sides of the animal by three-pronged alae that extend from head to tail (Fig. 1).

Transmission electron microscopy of the internal structure of the adult cuticle reveals two major cuticle layers separated by a space, presumably fluid filled. The outer cortical layer is composed of an electron dense surface layer and an amorphous under layer. The inner basal layer is composed of two fiber layers running in different direc-



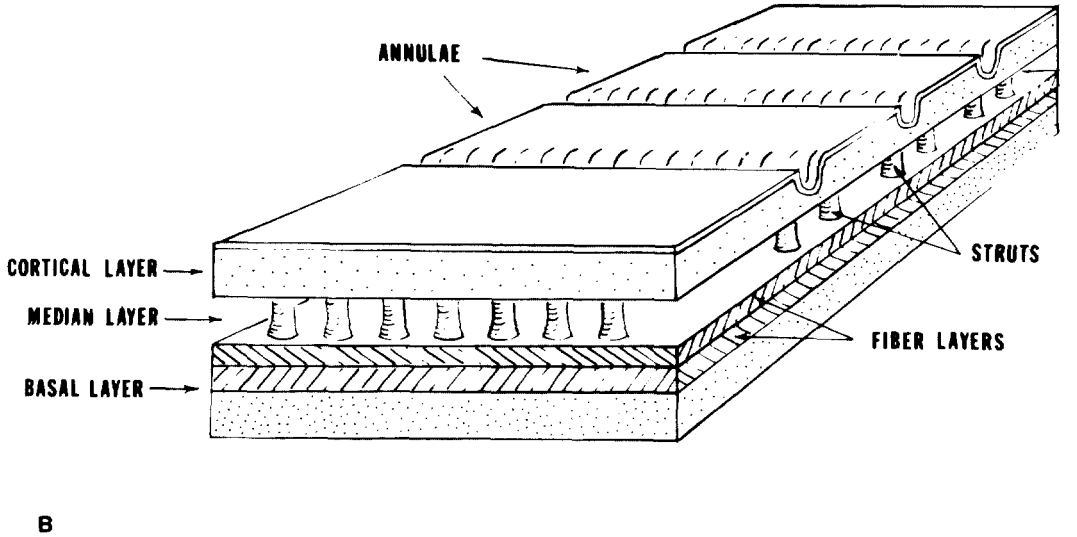


Fig. 1. Diagrammatic sketch of the adult *C. elegans* cuticle. (a) Cross-section of an adult nematode showing the general organization of the cuticle and underlying hypodermis. (b) Magnified view of internal anatomy of cuticle. Neither figure is drawn to scale. Reprinted from Cox *et al.* (4).

tions, probably helically about the animal, and an inner amorphous layer. The basal and cortical layers are joined by electron dense columnar structures we call struts; these run in circumferential paired rows under the annular furrows. Struts are also found irregularly between the paired rows. In the region of the alae, the basal and cortical layers appear fused, the struts are not evident, and the fiber layers appear unchanged. Many of these features of adult cuticle structure are evident in Figures 2 and 3 and are diagrammed in Figure 1.

The cuticle is an extracellular secretion of the hypodermis. The hypodermis in *C. elegans* is, for the most part, a syncytium (16). The nuclei are located in the lateral ridges; none are present in the dorsal or ventral ridges. These ridges are connected by a thin cytoplasmic bridge enclosing the muscle bundles. A row of hypodermal seam cells lies under each ala, 16 cells on each side; these cells fuse to form a seam cell synctium after the adult molt. Laser ablation of individual seam cells results in interruptions of the alae, indicating that the individual seam cells control the formation of the ala above them (15).

COMPOSITION OF ADULT

To prepare cuticles for biochemical study, we sonicate a suspension of animals to rupture the cuticle (3). Extensive washing of the cuticles in low ionic strength buffer removes most of the cellular material, but some contaminating material still remains. This can be removed by treatment with boiling detergent, such as SDS (sodium dodecyl sulfate), without major alteration in cuticle morphology, as determined by phase contrast light microscopy and transmission electron microscopy of SDS-cleaned cuticles. These cuticles show a slight birefringence. The direction of birefringence is at right angles to the long axis of the animal and presumably derives from the helically wound fiber layers that are oriented predominantly at right angles to the long axis of the animal. The birefringence of the alae is of opposite sign, indicating longitudinal orientation of material within the alae.

Along with the cuticle, these preparations contain the cuticular linings of the pharynx, uterus, anus, and excretory pore. Enzymatic digestion of isolated cuticles has been followed by phase contrast microscopy. Pronase and collagenase digest first the struts, to produce a double bag, and then the inner layer of the cuticle, but do not affect the outer layer. This outer layer of the cuticle is digested only by elastase which also digests the struts and inner layer. The pharyngeal lining is not digested by collagenase or elastase but is digested by pronase. These results suggest that the cortical layer, the struts and basal layer, and the pharyngeal lining all have different structural features that render them differentially sensitive to these three enzymes. We have found that treatment of the isolated cuticles with reducing agents such as β-mercaptoethanol (BME) gives results comparable to enzymatic digestion with collagenase: first solubilization of the struts, then the basal layer. The cortical layer, or a portion of it, is resistant to solubilization by BME.

Since cuticle integrity is little altered by hot detergents, cuticle proteins are held together by covalent bonds. The solubilization of the basal layer and the struts by BME suggests that the proteins composing these structures are held together by disulphide bonds. The insolubility in BME of a portion of the cortical layer suggests the existence of more extensive nonreducible covalent cross-linking of proteins in the cortical layer.

These observations are in accord with earlier studies on the cuticle of *Ascaris lumbricoides* (7,10) which revealed an inner layer composed of collagen-like proteins cross-linked by disulphide bridges and an outer layer composed of covalently cross-linked proteins that do not show the low angle X-ray pattern characteristic of collagens and so are inferred to be a different class of fibrous proteins, termed cuticlins.

The cuticle proteins solubilized by BME range in apparent molecular weight on SDS polyacrylamide gels from 60,000 to 210,000 daltons. Eight distinct species are reproducibly observed, as well as variable amounts of higher molecular weight material. The major species are 91,000 (28%) and 106,000 (47%) daltons. The other types range from 1% to 6% of the total solubil-

ized material. A similar complexity of protein species has also been observed for the Bergerac strain of *C. elegans* (11) and for another free-living nematode, *Panagrellus silusiae* (8). These results differ from those for *Ascaris lumbricoides* (6) where the cuticle appears to be composed of three different collagen polypeptides, each of molecular weight 52,000 daltons.

That the *C. elegans* BME soluble cuticle proteins are collagens or collagen-like is supported by several facts. All the cuticle proteins visualized on SDS gels stain pink rather than blue with Coomassie blue R-250, a characteristic of collagens. All are digested by purified bacterial collagenase, a protease that specifically recognizes the Gly-X-Y-Gly sequence. Pretreatment of the purified cuticles with pepsin at low temperature reduces somewhat the molecular weight of the cuticle proteins, but they are otherwise preserved from enzymatic attack. At low temperature, pepsin will not degrade the collagen triple helix, only the nonhelical regions. The amino acid composition of the soluble proteins resembles collagen. It is high in glycine (26%), proline (11%), and hydroxyproline (12%). The amino acid composition of the insoluble outer layer differs from that of the soluble layers, suggesting the presence of different noncollagen proteins in the outer insoluble component.

NUMBER OF PROTEINS

The great structural complexity of the cuticle suggests that it might be composed of a large number of different protein species whose special features contribute to the form of the cuticle. Collagens that make up the fiber layers might differ from those that compose the struts, for example. The form of the cuticle would then largely result from self-assembly of proteins secreted in a specified sequence and two dimensional pattern. The presence of a large variety of collagen species in the cuticle would not be surprising, since at least nine different collagens are present in vertebrate animals indicating that vertebrate collagens derive from a rather large gene family. Another extracellular structure, the insect chorion, is formed from the numerous protein products of an extensive gene family (12). Further, different chorion proteins are found in different component parts of the chorion.

Alternatively, one might imagine the cuticle to be largely composed of one or a small number of protein species aggregated together in different ways and modified by ancillary enzymes to produce morphological features of the cuticle. The complex internal architecture of the cuticle would then be a consequence of the way the components are assembled.

At present, we have little information that bears on this issue. There are at least eight different molecular weight forms of the soluble adult cuticle collagens. Many of these are made up of several forms separable in a second isoelectric focusing dimension. At least 18 cuticle proteins species are observed on two-dimensional gels. These could represent different gene products of an extensive gene family. However, they could also represent various secondary modifications of a much smaller group of proteins. The isoelectric variants could arise from glycosylation, for example. The different molecular weight species found might result from nonreducible covalent cross-linking of a small number of primary collagen polypeptides. We have demonstrated that some of the molecular weight species differ from each other in terms of peptides released by protease digestion, but this result might be found for different cross-linked aggregates as well as for different polypeptides.

We can isolate native molecules from the cuticle by sequential treatment of cuticle preparations by SDS and BME. SDS removes cellular debris while the cuticle proteins remain cuticle-bound through disulphide cross-links. If the SDS is now removed and the disulphides are reduced with BME in a buffered salt solution, native collagens are released from the cuticle. Presumably the triple helix is conserved by hydrogen bonds in the presence of BME. This was verified by the recovery of pepsin-resistant material from the cuticles using this procedure. Biochemical studies of such native molecules may help to clarify the extent of complexity of the cuticle proteins. Another, and perhaps quicker, approach to the problem of cuticle protein complexity may be to identify and characterize directly the cuticle collagen genes and mRNA's by gene

cloning techniques. We have found collagenase-sensitive proteins among the in vitro translation products of mRNA isolated from molting and adult *C. elegans*. In these RNA preparations, mRNA's of about 1 kb in size will hybridize to a DNA collagen probe, a plasmid with a cDNA insert derived from the $\alpha 2(I)$ collagen mRNA of the chick; these mRNA's are possibly the cuticle collagen messengers. J. Kramer, G. Cox, and D. Hirsh (personal communication) have used the chick collagen probe to clone several *C. elegans* DNA fragments (genes) that will hybridize to it. At least 15 such genes exist in *C. elegans*. Two of these genes have been partially sequenced and are found to be small, coding for proteins of molecular weight about 30,000 daltons. They contain interrupted stretches of collagen sequence (gly-X-Y-gly-X-Y). Whether these genes code for the cuticle proteins remains to be seen.

TEMPORAL REGULATION OF SYNTHESIS

Like other nematodes, *C. elegans* molts four times during its development from newly hatched L1 to adult. The L2, produced by molting from an L1, can in turn molt into either an L3 or a dauer larva, depending upon environmental conditions. Unlike the L3, the dauer can survive several months without food. When presented with food, it will resume the standard postembryonic developmental pathway by molting into an L4 and then into an adult. Except for the dauer to L4 transition, a molt that is triggered by food, the molts appear to be temporally rather than environmentally programmed.

At each molt, the old cuticle is shed and a new one secreted. We have followed the synthesis of this cuticle by pulse, and pulse-chase, labelling of protein with $NaH^{14}CO_3$ (2). This procedure has several advantages. The label rapidly enters the animal and can be chased out. It enters the amino acid pool through the tricarboxylic acid cycle, even during lethargus, when the animal is not feeding.

The labelling experiments were performed on populations of animals synchronized by triggering emergence from the dauer state with food. Our results show that a peak of cuticle protein synthesis coincides with the molts from dauer to L4 and L4 to adult. The proteins are newly synthesized at this time and old cuticle is not reutilized. During the peak of cuticle synthesis, about 10–15% of the proteins synthesized in the animal are cuticle proteins; the majority of the remaining labeled proteins are presumably gonadal. These experiments indicate that regulation of cuticle formation is at least controlled at the level of translation. Identification and study of cuticle protein mRNA's will be necessary to determine if regulation is transcriptional although indirect experiments in *Panagrellus* support that notion (13).

MORPHOLOGY, COMPOSITION/LARVAE

We have carried out a comparative study of various of the larval cuticles. The L1, dauer, and L4, as well as the adult cuticles, have been examined by transmission electron microscopy, and the proteins derived from these cuticles by SDS and BME extraction have been compared on SDS polyacrylamide gels. The cuticles of all these stages differ as well (5). Preliminary unpublished studies indicate that the cuticles of the L2 and L3 are very similar to the L4 cuticle in both morphology and protein composition.

Many of the morphological differences are revealed in Figures 2 and 3. The L4 cuticle has only one layer composed of an outer cortical region adjoined to two inner fiber layers. The dauer cuticle lacks fiber layers but has instead a basal striated region that appears as a box-like network in tangential section. The L1 cuticle also appears to have a basal striated region. The L2, L3, and L4 cuticles lack alae. The alae of the dauer and the L1 differ from one another and from the adult alae in form (Fig. 3). The L1 alae are composed of a large mushroom-shaped central lobe and two smaller lateral lobes, while the dauer alae are composed of five adjacent projections.

The types of extractable proteins released from the larval cuticles differ from one another (Fig. 4), although it should be noted that the various larval cuticles varied in the degree to which they were solubilized by our procedures. Nineteen different molecular weight species can be extracted from

one or other stage cuticle. Some of these protein species are unique to a given cuticle type, others are found in several stages. A few protein species are found in all stages. Some of the extractable larval proteins appear to be resistant to collagenase.

These results indicate that for some molts, the form and composition of the cuticle undergoes major transformation. *C. elegans* can make four different types of cuticles which overlap in architecture and protein components only to some extent. *C. elegans* differs from another free-living nematode, *Panagrellus silusiae,* which appears at each molt to make cuticles identical in morphology and protein composition (8, 14).

GENES AND MORPHOLOGY

The cuticle is the nematode's exoskeleton. As a consequence, it is not surprising that we have found most mutants that exhibit deformations in gross morphology display defects in cuticle architecture as well (4). These mutants are of various phenotypes: dumpy (a short fat animal), blister (fluid filled blebs in the cuticle), roller (helically twisted body, the animal rotates on its axis), and long (animal longer and thinner than wild type). In all, more than 35 genes can mutate to give one or other of these phenotypes. With many of the mutants, we have demonstrated derangements in internal cuticle architecture, although as yet no systematic electron microscopy has been done. Alterations in gel patterns are observed for extractable proteins from one type of blister mutant, but the results are preliminary.

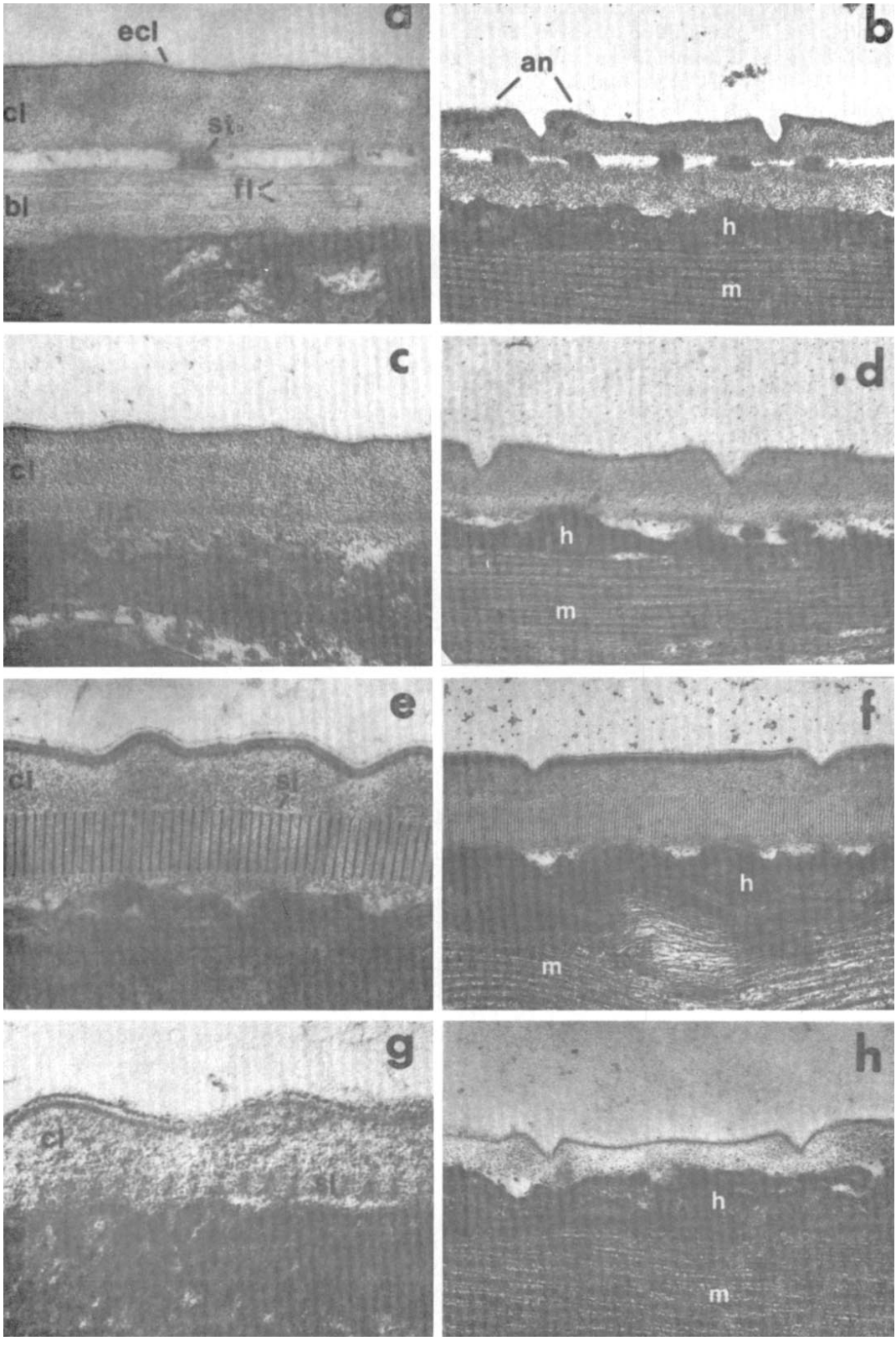
Mutants defective in five of the six blister genes exhibit a mutant phenotype only in animals with an adult cuticle; larvae are normal. Our observations are consistent with the notion that mutations in these five genes result in strut defects which, in turn, lead to formation of blebs between the cortical and basal layer of the adult cuticle. The larvae have cuticles of different anatomy that lack struts and so are normal in phenotype.

The roller mutants fall into two classes: those that are twisted helically either to the right or to the left. Our observations are consistent with the notion that the two classes of genes (right roller and left roller) control the formation of the two fiber layers of the cuticle. Many of these roller mutants also display stage specificity in the expression of mutant phenotype; some mutants ro'l only as adults, others only as dauers or L3's, for example. Dumpy mutations are very frequent and are located in 1 of 22 or more different genes. Many dumpies have major alterations in cuticle morphology and most do not display a stage-specific mutant phenotype. Preliminary electron microscope analysis of a morphological mutant termed *squat* reveals an adult cuticle apparently defective in the *sequence* in which cuticle components are secreted. The strut material is located, as expected, under the annular furrows, but imbedded between the fiber layers, rather than joining the basal and cortical layers. SDS gels of this mutant reveal a cuticle of normal protein composition.

The above result illustrates the difficulty of determining the nature of the primary gene function for each of these 35 genes. Some may be structural genes for a stage-specific cuticle protein. Others may control the secretion of component proteins. Still others may control enzymes involved in post-translational modifications of cuticle proteins. The results we have so far suggest that there are a large number of genes involved in cuticle formation, and some of these genes are stage specific and therefore active in assembly of one of the four cuticle types that *C. elegans* can form. Other genes appear to act at all stages of cuticle formation.

GENE REGULATION OF FORMATION

Several genes of *C. elegans* have been discovered that may be major regulatory genes controlling transitions from one post-embryonic stage to another (V. Ambros and R. Horvitz, personal communication, 1). These genes were first identified from mutants with phenotypic alterations in post-embryonic cell lineage patterns. Mutants defective in the gene *lin-4* appear to reiterate the L1 cell lineage pattern after each molt and have a larval-type cuticle even as a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sexually mature adult. We have verified that sexually mature *lin-4* mutant adults have an L2 type cuticle (L2, L3, and L4 are all very similar) as identified by the gel pattern of the extractable proteins. Note that since an L1 animal makes an L2 cuticle, one would expect an animal that reiterates the L1 state to make an L2 cuticle. We have also shown that animals containing a *lin-4* mutation together with an adult specific blister mutation fail to develop blisters, presumably because they never produce an adult type cuticle, the only one susceptible to a blister phenotype. Some mutants defective in another gene, *lin-14,* apparently skip the L1 stage and precociously develop an adult type cuticle after the third rather than fourth molt (V. Ambros and R. Horvitz, personal communication).

The existence of these mutants suggests that the transition from one stage to another is a major transformation involving changes in at least cuticle forming capacity and cell lineage patterns. If so, the genes *lin-4* and *lin-14* may be part of a genetic regulatory system controlling the transitions from stage to stage. The changes in cuticle morphology that accompany some of the molts may prove useful as a diagnostic probe for identifying additional genes that control these events. One could, for instance, use blister mutants to look for secondary mutations that cause blisters (and therefore adult cuticles) to form during larval molts.

SPECULATIONS

The *C. elegans* cuticle is a complex extracellular structure whose architecture and protein composition is altered drastically during some of the post-embryonic molts. Many genes are involved in the formation of the cuticle, and some of these genes are stage specific in their activity, acting at some but not other of the post-embryonic molts. The existence of mutations that alter the phenotype of various post-embryonic stages so that patterns of cell division and differentiation and also cuticle formation are reiterated or skipped suggests that a molt is not simply a mechanism for shedding a cuticle and making a larger one to accommodate growth, it is more of a metamorphosis.

A distinctive phylum characteristic of nematodes is the tremendously varied and complex life cycles developed by many parasitic forms. In general, the change from one habitat to another (for instance, the transition from an encysted stage in the muscle of a cockroach to the free-living in the gut of a bird) is coincident with a molt. Conceivably, the evolutionary adaptive radiation of nematodes occurred as a consequence of the ability of members of the phylum to evolutionarily modify the adaptive features of each molt stage independently of the others, allowing for sequential transformations suiting the animal to very different ecological niches at different stages of its development.

We point to an analogous situation in the annelids and arthropods where morphological segmentation was followed by acquisition of the capacity to evolutionarily modify segments independently of each other. This characteristic of arthropods may well have contributed to their adaptive radiation. The study of genes that control homeotic transformations of segments in *Drosophila melanogaster* is contributing to our understanding of genetic control of arthropod development (9). Perhaps analysis of genes like *lin-4* that cause homeotic transformations of sequential temporal stages in nematode development will be equally interesting.

The finding of four different cuticle types during post-embryonic development of *C. elegans* requires explanation. Possibly *C. elegans* is derived from a parasitic ancestor and so retains vestiges of some parasitic cuticular adaptations in the morphology of its larval cuticles. Alternatively, *C. elegans*

Fig. 2. Transmission electron micrographs of adult and juvenile cuticles. Transverse (left column) and longitudinal (right 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 annulae (an), basal layer (bl), cortical layer (cl), external cortical layer (ecl), fiber layer (fl), striated layer (sl), and struts (st). Hypodermal (H) and muscle (M) cells are also shown. Reprinted from Cox *et al* (5).

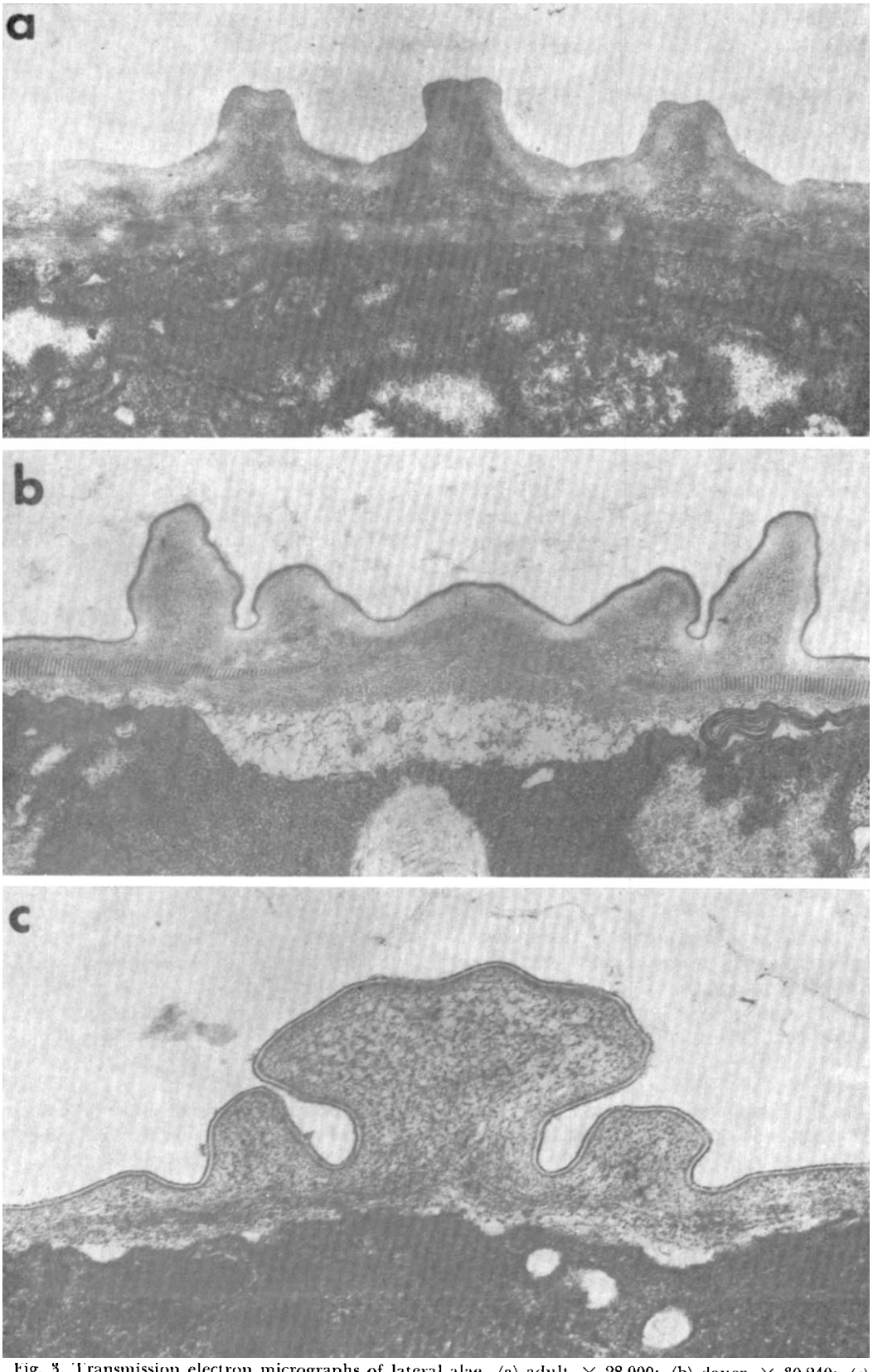

Fig. 3. Transmission electron micrographs of lateral alae. (a) adult, × 28,000; (b) dauer, × 30,240; (c) L1, × 84,000. Reprinted from Cox *et al* (5).

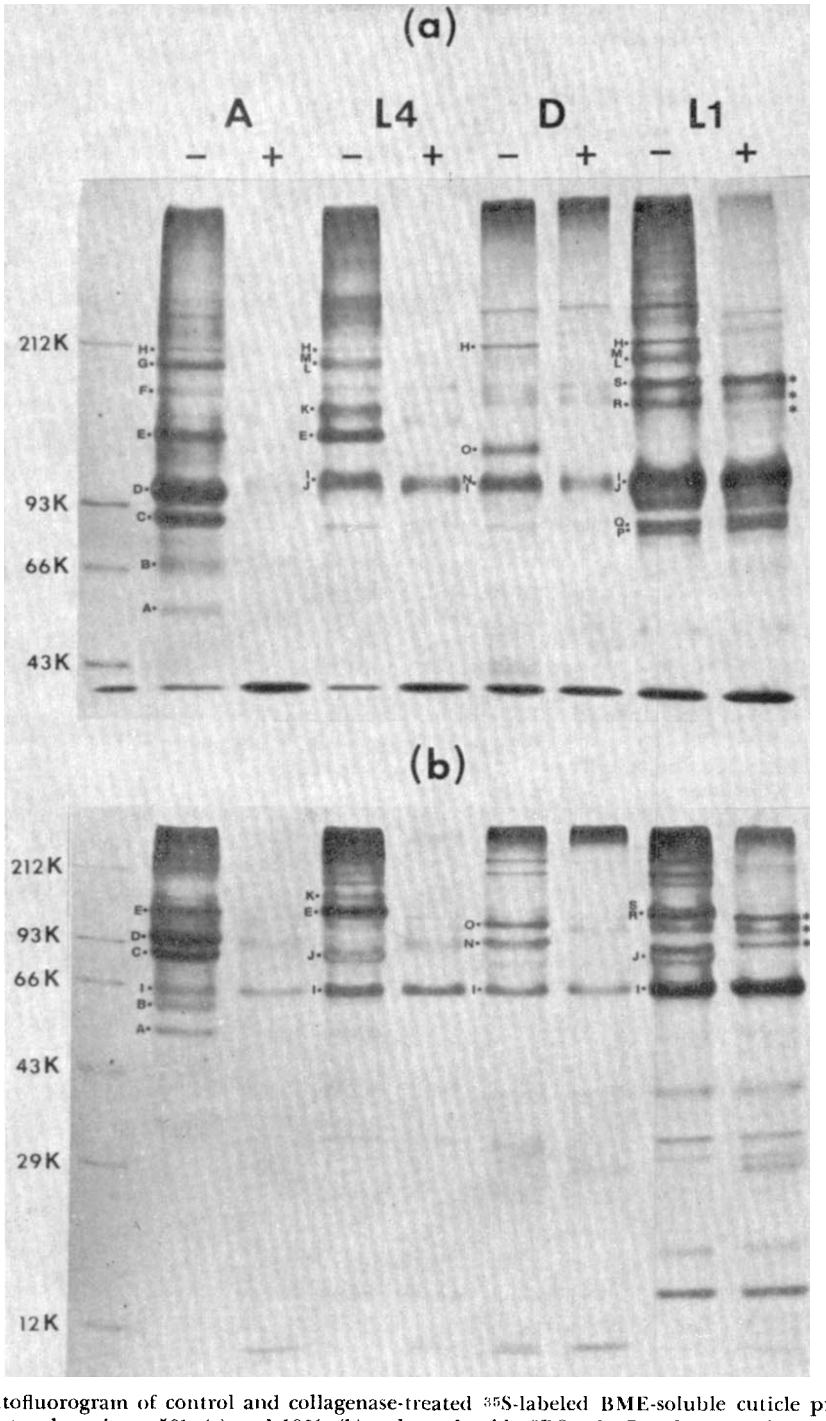

Fig. 4. Autofluorogram of control and collagenase-treated ^{35}S-labeled BME-soluble cuticle proteins separated by electrophoresis on 5% (a) and 10% (b) polyacrylamide SDS gels. Developmental stages are given above gel lanes (A, adult; D, dauer) and approximate molecular weights of protein standards are indicated in left column. Major cuticle proteins are identified with capital letters. Reprinted from Cox *et al.* (5).

might be a facultative parasite whose host has not yet been found. In support of *C. elegans* being derived from a facultative parasite ancestor is our observation that some cuticle mutants have different adult phenotypes depending on whether the adult is derived from a dauer or L3 larva (4). Although it has been customary to view the

dauer as simply an alternative to L3, this observation suggests that the post-embryonic developmental pathway is branched: the adult developing from a dauer differs at least in some respects from an adult developing from an L3.

While some, or indeed all, of these speculations may prove to be wrong, we hope they will serve to stimulate further study of post-embryonic development in *C. elegans* and its genetic control.

LITERATURE CITED

1. Chalfie, M., R. Horvitz, and J. Salston. 1981. Mutations that lead to the reiterations in the cell lineages of Caenorhabditis elegans. Cell 24:59-69.

2. Cox, G. N., M. Kusch, K. DeNevi, and R. S. Edgar. 1981. Temporal regulation of cuticle synthesis during development of Caenorhabditis elegans. Develop. Biol. 84:277-285.

3. Cox, G. N., M. Kusch, and R. S. Edgar. 1981. The cuticle of Caenorhabditis elegans. Its isolation and partial characterization. J. Cell Biol. 90:7-17.

4. Cox, G. N., J. S. Laufer, M. Kusch, and R. S. Edgar. 1980. Genetic and phenotypic characterization of roller mutants of Caenorhabditis elegans. Genetics 95:317-339.

5. Cox, G. N., S. Staprans, and R. S. Edgar. 1981. The cuticle of Caenorhabditis elegans. II. Stage specific changes in ultrastructure and protein composition during post embryonic development. Develop. Biol. 86:456-470.

6. Evans, H. J., C. E. Sullivan, and K. A. Piez. 1976. The resolution of Ascaris cuticle collagen into three chain types. Biochemistry 15:1435-1439.

7. Fujimoto, D., and S. Kanaya. 1973. Cuticlin: a noncollagen structural protein from Ascaris cuticle. Arch. Biochem. Biophys. 157:1-6.

8. Leushner, J. R. A., N. E. Semple, and J. P. Pasternak. 1979. Isolation and characterization of the cuticle from the free-living nematode Panagrellus silusiae. Biochim. Biophys. Acta 580:166-174.

9. Lewis, E. B. 1978. A gene complex controlling segmentation in Drosophila. Nature 276:565-570.

10. McBride, O. W., and W. P. Harrington. 1967. Ascaris cuticle collagen: on the disulfide crosslinkages and the molecular properties of the subunits. Biochemistry 6:1484-1498.

11. Ouzana, R., and D. Herbage. 1981. Biochemical characterization of the cuticle collagen of the nematode Caenorhabditis elegans. Biochim. Biophys. Acta 669:236-243.

12. Regier, J. C., G. D. Mazur, and F. C. Kafatos. 1980. The silkmoth chorion: morphological and biochemical characterization of four surface regions. Develop. Biol. 76:286-304.

13. Pasternak, J., and J. R. A. Leushner. 1975. Programmed collagen synthesis during postembryonic development of the nematode Panagrellus silusiae: Effect of transcription and translation inhibitors. J. Exp. Zool. 194:519-528.

14. Samoiloff, M. R., and J. Pasternak. 1968. Nematode morphogenesis: fine structure of the cuticle of each stage of the nematode Panagrellus silusiae. Can. J. Zool. 46:1019-1022.

15. Singh, R. N., and J. E. Sulston. 1978. Some observations on molting in Caenorhabditis elegans. Nematologica 24:63-71.

16. White, J. G. 1974. Computer-aided reconstruction of the nervous system of Caenorhabditis elegans. Ph.D. Thesis, University of Cambridge, Cambridge, England.

17. Zuckerman, B. M., S. Himmelhock, and M. Kisiel. 1973. Fine structure changes in the cuticle of adult Caenorhabditis briggsae with age. Nematologica 19:109-112.