Nematologica 24 (1978): 63-71. E. J. Brill, Leiden

SOME OBSERVATIONS ON MOULTING IN *CAENORHABDITIS ELEGANS*

BY

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Moulting of *Caenorhabditis elegans* has been observed by Nomarski interference contrast microscopy, and by electron microscopy of animals at selected stages. The wild type, cell division mutants and animals in which cells had been ablated by a laser microbeam were examined. The median lateral hypodermis, or "seam", is required for the formation of alae and for dauer larva maturation. During cuticle deposition, large Golgi bodies are seen in the seam cells. The excretory system is not essential for moulting.

Using the technique described by Sulston & Horvitz (1977), it is possible to observe a single specimen of *Caenorhabditis elegans* throughout its life cycle, without the use of anaesthetics, by high resolution Nomarski interference contrast microscopy. Thus, events can be followed in their natural succession and animals can be removed at any stage for electron microscopic examination. A laser microbeam system and genetic methods allow experimental manipulation of the organism.

In describing moulting, we shall be concerned to a great extent with the hypodermis, which both generates and underlies the cuticle. The structure of the hypodermis of *C. elegans* has been elucidated by White (1974). It consists of four longitudinal ridges (ventral, dorsal and lateral) joined circumferentially by thin sheets of cytoplasm, and can be divided into two parts. The greater part comprises a single large syncytium which extends throughout most of the animal, and a series of smaller syncytia and individual cells in the head and the tail. In young larvae, the lesser part comprises two longitudinal rows of "seam" cells which form the median lateral hypodermal chords. Seam cells are rigorously defined by the special role which they play in cuticle secretion, as described below. At the L4 larval stage the seam cells of each side fuse together to form a continuous band; until this time most of them act also as hypodermal stem cells (Sulston & Horvitz, 1977).

The term "pharynx", which will be used in conformity with current *C. elegans* literature, is synonymous with "oesophagus".

MATERIALS, METHODS, NOMENCLATURE

Caenorhabditis elegans (var Bristol, strain N2) was cultured as described by Brenner (1974). For Nomarski microscopy, animals were mounted between

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an agar slab and a cover slip as described by Sulston & Horvitz (1977); photography was by microflash. For electron microscopy, selected individuals were transferred into 1% osmium tetroxide in 0.1M phosphate buffer at pH 7.4, and left for one hour at room temperature; subsequently, the procedure of Ward *et al.* (1975) was followed. For the demonstration of pharyngeal granules, the head was first fixed in 2% glutaraldehyde for 2 hours (Wolff *et al., in litt.*).

A laser microbeam system developed by J. G. White was used to kill individual cells. It is based on a pulsed coumarin dye laser (Berns, 1972) and produces a focussed spot about 1.5 μm in diameter. Before surgery, nematodes were mounted on agar containing the anaesthetic 1-phenoxy-2-propanol (0.2%-0.4%).

Methods for isolation and characterisation of mutants have been described by Brenner (1974). E 1348 (lin-5 II) and E 1466 (lin-6 I) are sterile recessive mutants which are maintained as heterozygotes (Albertson, Horvitz, Sulston, White, unpubl.). The young L1 appears normal, possibly because sufficient wild type gene products for embryogenesis are inherited from the heterozygous mother. In E 1348, somatic cell division is almost completely blocked after hatching and germ cell division is abnormal after the L1, but DNA replication continues. The seam cells become large and polyploid, and gradually fuse with the large hypodermal syncytium, perhaps as a consequence of expressing the character which their anterior daughters possess in the wild type (Sulston & Horvitz, 1977). In E 1466 there is little or no somatic DNA replication after hatching; cell division continues, however, and the seam cells and the other blast cells atrophy or die.

Ecdysis is defined as the moment at which the head of a larva breaks out of the old cuticle; lethargus is the period of inactivity preceding ecdysis; moult is used as a general term for the entire process. Moult, lethargus and ecdysis are named for the larval stage which they terminate: e.g. L4 ecdysis converts an L4 larva into an adult..

RESULTS

General. In common with other nematodes, *C. elegans* sheds its cuticle four times during development. Each ecdysis is preceded by a period of lethargus, during which pumping and locomotion are suppressed; lethargus usually lasts for about 2 hours. The cuticles of certain stages are distinguished by bearing alae. On each side, the L1 larva has a small double ala, the adult has three small alae, and the dauer larva has a broad five-fold ala; the L2, L3 and L4 larvae have no alae.

All moults have been observed repeatedly by Nomarski microscopy. Those of the normal life cycle appear qualitatively similar, and a single comprehensive description is given; the moults at entry to and exit from the dauer larva differ from the normal moults in certain respects and are treated separately. Hermaphrodites at selected stages in the L3, L4 and pre-dauer states have been examined by electron microscopy.

The normal moult. During the first part of the intermoult period, the hypodermal cytoplasm appears relatively smooth by Nomarski microscopy. Only a few

small Golgi bodies are seen in electron micrographs, there is no sign of new cuticle beneath the old, and the cytoplasm appears inactive (Fig. 2A).

Between 2 and 4 hours before lethargus, the cytoplasm of the seam cells becomes coarsely granular as observed by Nomarski microscopy (Fig. 3); the rest of the hypodermis remains comparatively smooth, although sometimes elongated blobs can be seen. Electron microscopy reveals that the granularity is due to densely packed large Golgi bodies in the seam cells (Fig. 2C); a few Golgi bodies are found elsewhere, but they are smaller and more sparsely distributed. The appearance and disappearance of the large Golgi bodies coincides with the appearance and disappearance of Nomarski granularity (Fig. 1), and the diameter of the

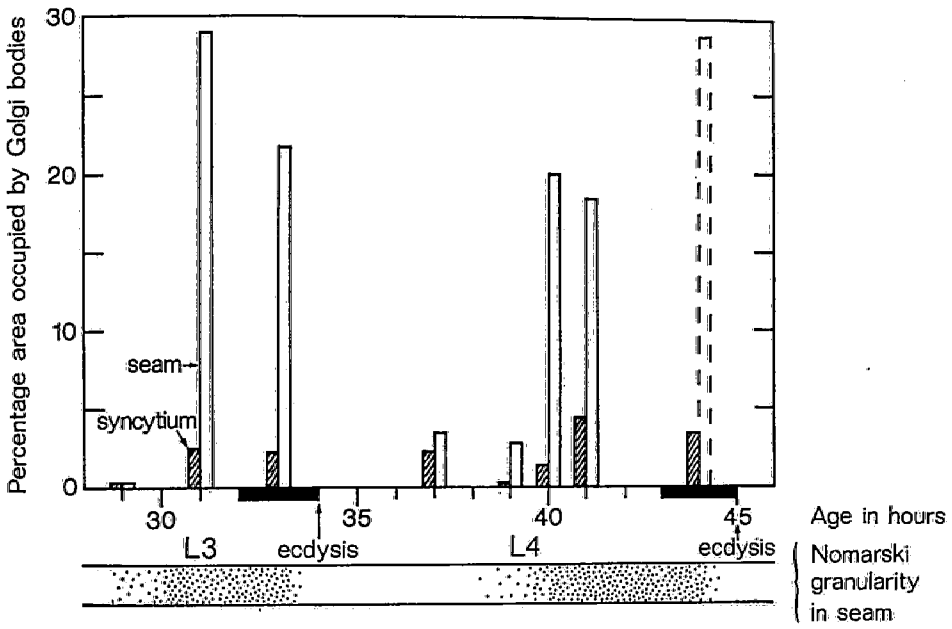

Fig. 1. Cyclic Golgi activity in hypodermis. Lethargi marked as black bars on abscissa. Ages of nematodes estimated either by timing from previous ecdysis or by reference to cell lineages (Sulston & Horvitz, 1977). Relative area occupied by Golgi bodies determined by measurement on ten to twenty electron micrographs. Plain bars: percentage area occupied by Golgi bodies in the seam; hatched bars: maximum percentage area occupied by Golgi bodies in the large hypodermal syncytium (3 μm^2 region most densely packed in each section). High value at 44hr is misleading, because the seam has shrunk greatly.

Golgi bodies (0.6-1.2 μm) is the same as that of the granules. In electron micrographs (Fig. 2C) the dilation of the cisternae indicates increased synthetic activity; vesicles containing darkly staining material appear to be budding off from the Golgi bodies and moving to the surface of the cell where the new cuticle is being deposited.

At a slightly earlier stage, in the L4 larva only (Fig. 2B), the seam is seen to be forming the alae of the adult cuticle; at this stage the nascent alae are clearly

visualised by Nomarski microscopy also. The following observations confirm that the seam is necessary for the formation of alae: —

1) When several seam cells are killed in the L3 or young L4, by the use of the laser microbeam, a gap is visible in the seam at L4 lethargus. A corresponding gap appears in the alae of the adult cuticle (Fig. 4). When the sister cells, whose presumptive fate is to fuse with the large hypodermal syncytium, are killed, there is no gap in either the seam or the alae.

2) In the mutant E 1348, there is usually no sign of the seam at L4 lethargus and no adult alae are formed. By chance, however, the formation of a short length of alae was noticed in one individual, and was seen to coincide precisely with a region of normal seam activity.

At the onset of lethargus, pumping and locomotion decrease gradually over a period of about 15 minutes, with occasional bursts of activity. At the same time, the old cuticle becomes loosened from the tip of the head and sealed across the mouth (Fig. 5). As lethargus proceeds, it also becomes loose in the buccal cavity and around the tail, but elsewhere the gap between the old and new cuticles is no more than a fraction of a micrometre. During lethargus the seam cells lose their granular appearance but, except in the L4, remain swollen in preparation for division at about the time of ecdysis.

During the second half of lethargus, animals often "flip" repeatedly; that is to say, they turn over by rotating rapidly 180° about the longitudinal axis. Such flipping is a distinctive feature of lethargus and probably helps to loosen the old cuticle. It does not normally occur during intermoult periods, but is sometimes seen after abuse such as handling or refrigeration. Flipping seems to be initiated in the head; this is consistent with the fact that only in the head are the left and right muscle quadrants separately innervated (White *et al.*, 1976), thus allowing torque to be generated at a bend in the animal.

About 30 minutes before ecdysis the posterior bulb of the pharynx begins to twitch spasmodically, spontaneous body movements begin, and large refractile granules are seen accumulating in the three "gl" gland cell bodies of the pharynx. [The anatomy of the pharynx has been described by Albertson and Thomson (1976). The gl cell bodies lie in the posterior bulb of the pharynx and their processes run forwards; the dorsal gland opens just behind the buccal cavity, and the two sub-ventral glands open just behind the anterior bulb.] About 10 minutes before ecdysis the granules move forward through the gl processes (Fig. 6A) and the entire pharynx begins to contract spasmodically. The cuticular lining of the pharynx breaks and the posterior piece is passed into the intestine. Meanwhile, the old cuticle is inflated around the tip of the head; the nematode pulls back from it repeatedly (Fig. 7) until the remainder of the pharyngeal lining is detached and then expelled by shuddering movements of the pharynx. If the nematode fails to expel the anterior piece at this point, it will be left with a cuticular plug in its mouth after ecdysis (Fig. 8); such a plug is not easily dislodged and occasionally causes death by starvation. Once its mouth is free, the nematode

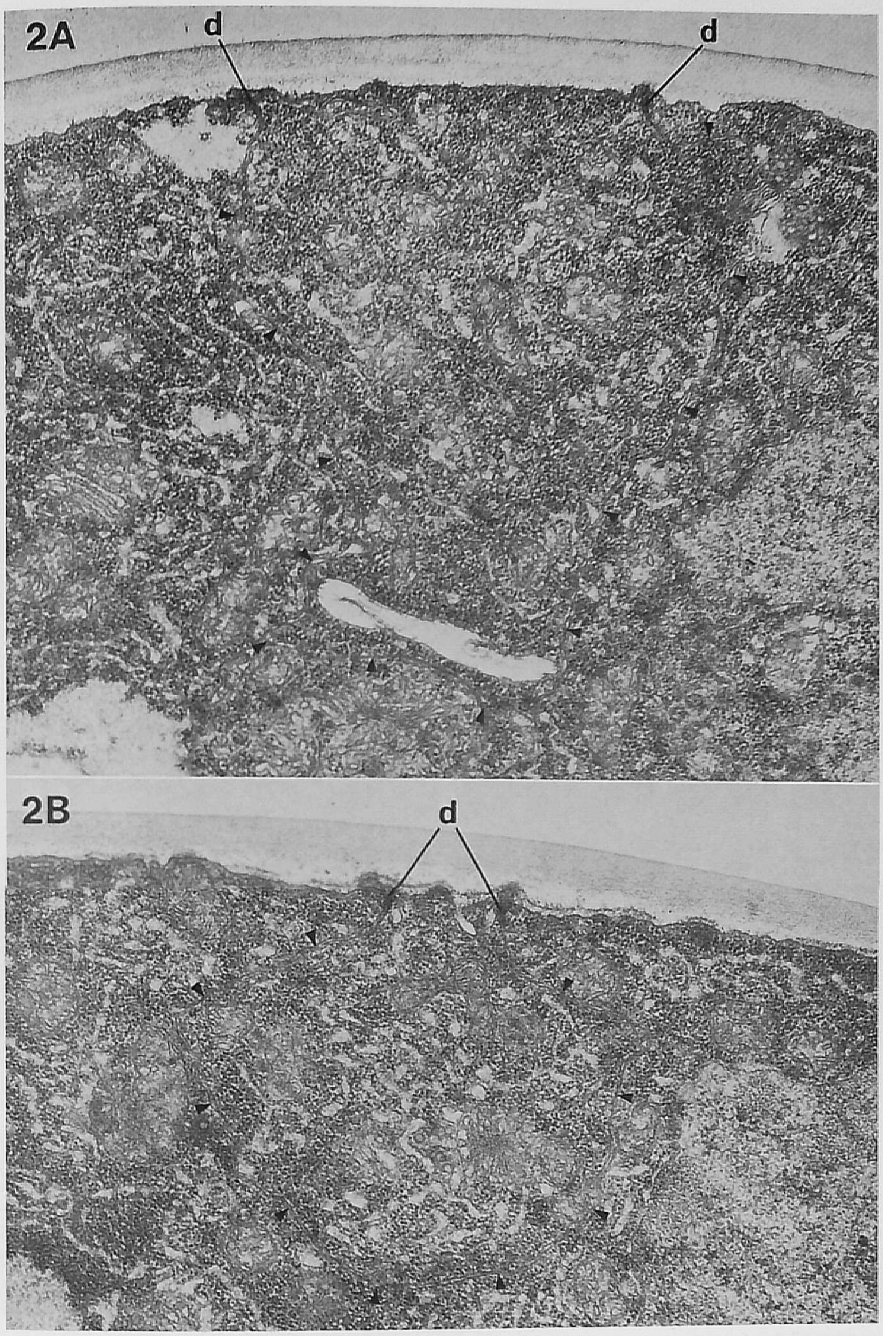

Fig. 2. L4 larvae, lateral hypodermis, transverse section, × 25,000. Superficial junctions of seam with large hypodermal syncytium marked by desmosomes (d); arrows show outline of seam. A) Age about 37hr. Hypodermis relatively inactive; a few small Golgi bodies outside seam. B) Age about 39hr. Hypodermal cisternae expanding; adult cuticle being deposited, with alae over seam. C) Age about 41hr. Seam packed with large Golgi bodies.

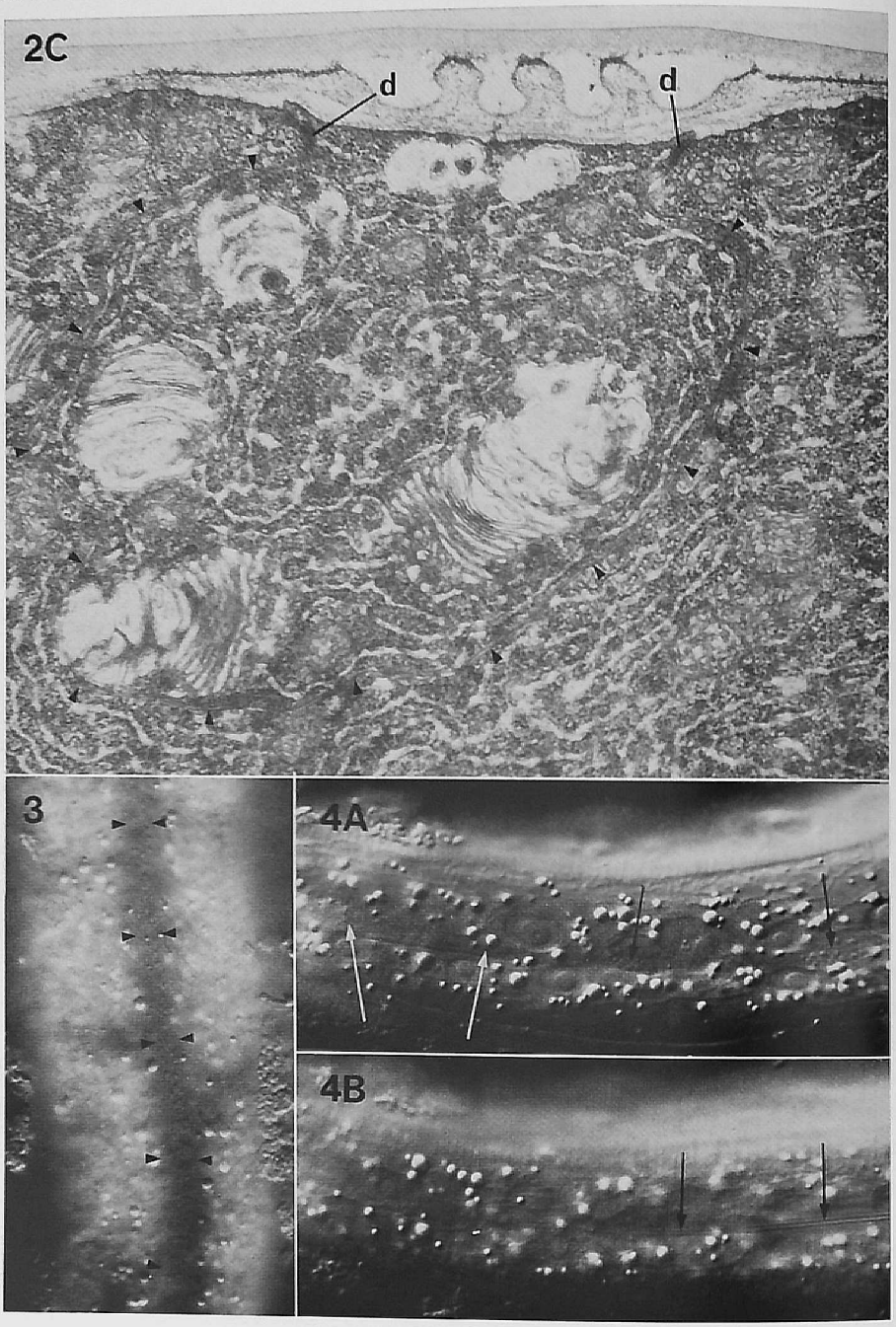

Fig. 3. L4 larva, lateral view, hypodermal plane of focus, Nomarski × 1000. Seam (arrowed) lies approximately over dark intestinal lumen. Golgi bodies (within the seam) are distinct from more refractile storage granules.

Fig. 4. Seam cells V5ppppp and V6pappp (Sulston and Horvitz, 1977) were ablated in a young L4 by the laser microbeam (white arrows). At L4 lethargus corresponding regions of seam and alae were absent. Nomarski × 1000. A) Hypodermal plane of focus; black arrows show regions of normal seam activity. B) Adult cuticle plane of focus; black arrows show normal alae.

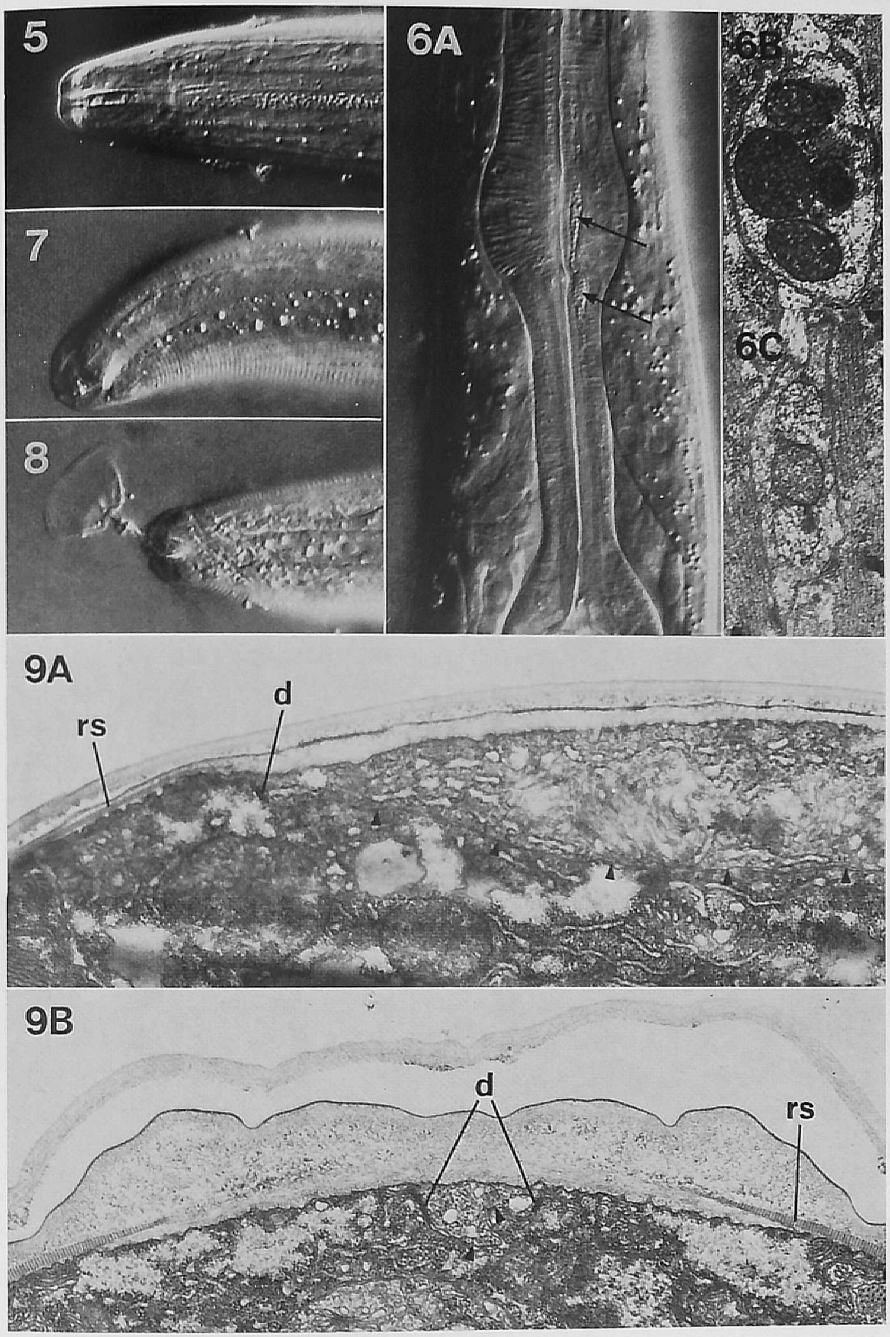

Fig. 5. Cuticle loose and sealed across mouth in early L3 lethargus. Nomarski ×1000.
Fig. 6. A) Large granules (arrowed) moving through dorsal pharyngeal gland shortly before L4 ecdysis. Nomarski × 1000. B) Large granules and C) Small granules in dorsal pharyngeal gland of L4, glutaraldehyde fixation, × 25,000.
Fig. 7. L3 larva pulling back on old cuticle shortly before ecdysis. Nomarski × 1000.
Fig. 8. L4 larva: mouth plug resulting from failure to dislodge pharyngeal lining before L5 ecdysis. Nomarski × 1000.
Fig. 9. Dauer formation; lateral hypodermis, transverse section, × 20,000; rs: radial striations; seam arrowed. A) Early cuticle deposition; only part of seam included in photograph. B) Stage of shrinkage.

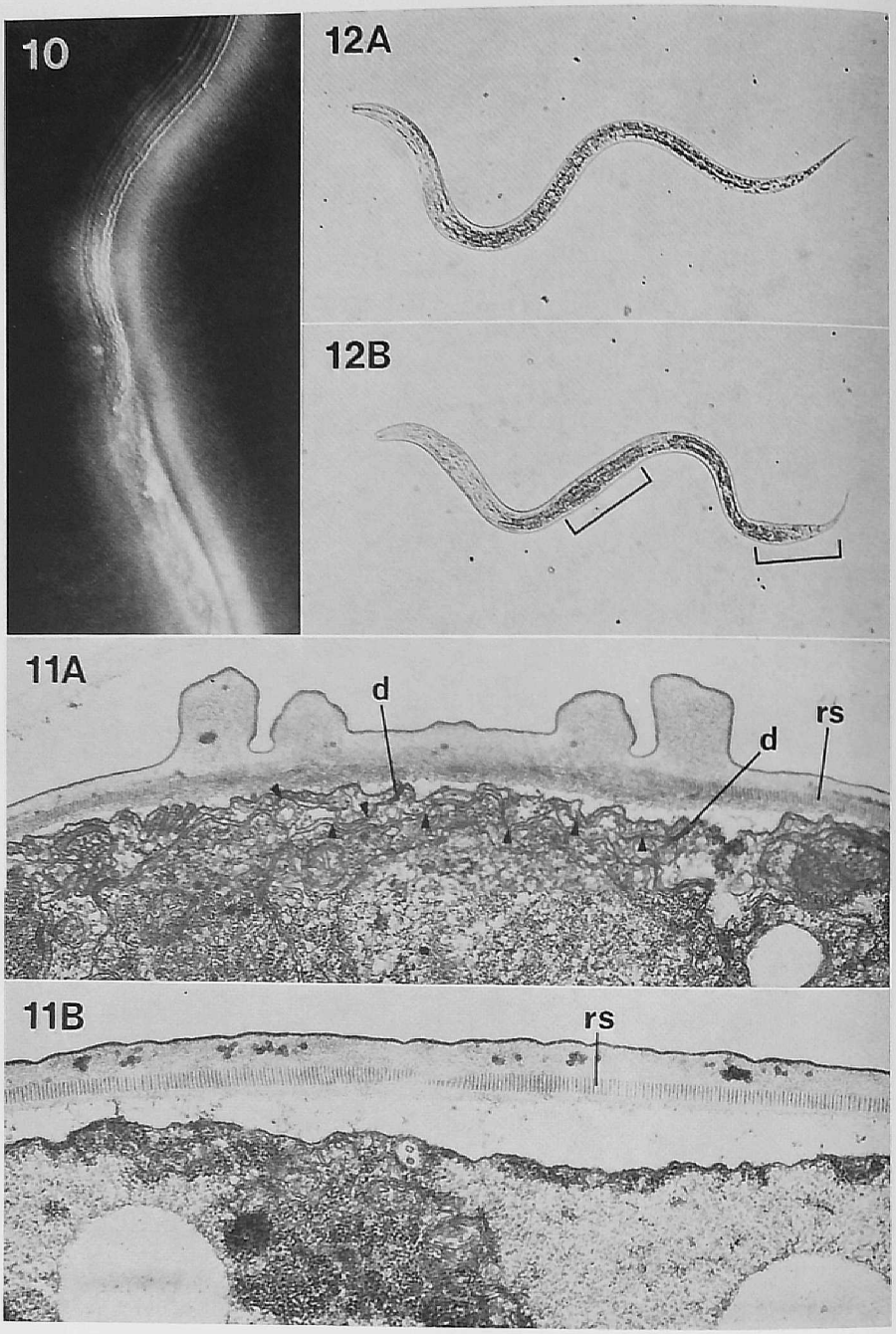

Fig. 10. E 1348 dauer larva, lateral view showing incomplete alae. Nomarski × 1000.
Fig. 11. E 1348 dauer larva, lateral hypodermis, transverse section, × 25,000. A) Region with seam and alae. B) Region with neither seam nor alae.
Fig. 12. Dauer larvae, diffuse illumination × 200. A) Wild type. B) E 1348 showing variable diameter. Alae absent from bracketed regions.

pushes against the old cuticle with its head until either a hole is made or a cap breaks away. Feeding begins immediately and, during the next few minutes, the nematode crawls out of its old cuticle.

The level of activity observed in the pharyngeal glands prior to ecdysis is not approached at any other time. During intermoult and early lethargus the granules in the dorsal gland are smaller: their diameters (measured on Nomarski and electron micrographs, see Fig. 6) are 0.2-0.8 μm in the pre-ecdysis burst and 0.15-0.3 μm at other times. The sub-ventral glands usually contain a few large irregularly shaped granules during feeding.

There is also a distinctive period of pharyngeal gland activity beginning about one hour before hatching. This is followed by spasmodic pumping; the pharyngeal lining is swallowed and what appears to be a vestigial stylet plugging the mouth is lost. The external cuticle is not shed, but the egg shell becomes softer, yielding to the thrusting of the larva, and eventually ruptures. Thus, the pharyngeal lining is shed five times during development, corresponding to the loss of one egg shell and four external cuticles.

The dauer larva. When their food supply is restricted, the nematodes pass through a specialised L2 moult and develop into dauer larvae in place of the normal L3 larvae (Cassada & Russell, 1975).

A number of L2 larvae were picked from an old plate and examined by Nomarski and electron microscopy. An early stage in the formation of the dauer cuticle is shown in Fig. 9A. The seam is circumferentially extended and contains large Golgi bodies; over it, the new cuticle is especially thick and lacks the radial striations which are appearing elsewhere. Dilated cisternae indicate synthetic activity in the hypodermis.

The entry into the dauer state can be watched by mounting individuals at early L2 lethargus on agar without bacteria; when so mounted before lethargus, the larvae usually escape. Electron microscopy at this stage reveals small Golgi bodies in the syncytial hypodermis; the seam is reduced and lacks Golgi bodies. The new cuticle is about 0.4 μm thick, except laterally where it is ridged up to 0.7 μm. The characteristic radial striations of the dauer cuticle are fully developed; laterally, they give way to circumferential fibres. After an hour or so, body movements begin, but there is no ecdysis at this point. Instead, the lips gradually clamp together, breaking off the cuticle in the buccal cavity. The animal shrinks about 10% in diameter, and to some extent in length, leaving a fluid filled space between it and the L2 cuticle; this loss of water might account for the relatively high density of dauer larvae (Cassada & Russell, 1975). Meanwhile the animal flips repeatedly within the L2 cuticle, pulling out the linings of the rectum, excretory duct and sensilla. As it shrinks, the alae become visible. At first they crumple with the body movements and the animal is free to bend in any direction, but after half an hour they remain stiff and the animal bends only dorsoventrally. Electron micrographs of a shrinking dauer (Fig. 9B) show a very small seam and little

synthetic activity in the hypodermis. The cuticle is thinner than before (about 0.25 μm), except laterally where it has formed the alae (up to 1.0 μm).

The loose L2 cuticle is usually lost eventually, but this process has not been watched.

Dauer larvae of the mutant E 1348 frequently have incomplete alae (Fig. 10). Two such larvae were examined by electron microscopy. In both, alae were found only over and slightly beyond intact seam cells (Fig. 11). A slight discrepancy is not surprising, since the seam may well have continued to diminish after ala formation. Dauer larvae of E 1466 also have incomplete alae. We conclude that the seam is necessary for ala formation in dauers.

Three further points emerge from the study of E 1348 and E 1466 dauer larvae. Firstly, the diametric shrinkage which normally accompanies dauer formation occurs little or not at all in regions lacking alae and, by implication, seam cells (Fig. 12). Secondly, in regions without alae the lateral hypodermis is like dorsal and ventral hypodermis: the circumferential fibres are replaced by radial striations, which are continuous except for a slight mid-lateral discontinuity (Fig. 11B). Thirdly, the larvae are much less resistant to 1% sodium dodecyl sulphate (SDS) than their heterozygous siblings. Cassada & Russell (1975) have shown that SDS resistance arises several hours after entry to L2 lethargus; perhaps the shrinkage brings about, or is a necessary prerequisite for, reduced permeability of the cuticle.

When exposed to bacteria, dauer larvae soon begin to feed and eventually moult into normal L4 larvae (Cassada and Russell, 1975). The dauer moult is similar to a normal L3 moult, differing only in the breaking of the old cuticle at a relatively well defined point at about the level of the excretory duct. In one individual the tear was seen to originate at the duct itself, and to propagate circumferentially; this may be the usual mode of ecdysis. However, as in normal moults, the loosening of the old cuticle begins at the mouth rather than at the excretory duct.

Mechanism of ecdysis. In contrast with the behaviour of the pharyngeal glands, the excretory glands do not display obvious cyclic activity. Their content of refractile granules, which are smaller than the ones seen at ecdysis in the pharynx but are still clearly resolved by Nomarski optics, increases during development and is at a maximum in the adult. However, because of the proposed involvement of the excretory system in moulting of some nematodes (Bird, 1971), we have explored its role in the ecdysis of *C. elegans* by killing cells with the laser microbeam. These experiments are not easy, because destruction of the large cells of interest requires energetic pulses which cause unwanted damage, and animals often take some time to recover.

a) Excretory glands. Two L2 larvae and two dauer larvae developed into normal adults after complete destruction of both excretory glands. A number of others matured with one gland surviving but forming no visible granules.

b) Excretory cell and duct. One L2 developed into a small adult after destruction of the excretory cell; some eggs were laid, but only about half of them hatched. One dauer developed into a small sterile adult after destruction of both the excretory cell and the duct cell, and the subsequent atrophy of the excretory glands.

c) gl glands of pharynx. It has not yet proved possible to destroy these cells without causing so much damage to the pharynx as to make the experiment meaningless.

After the operations the process of moulting was not usually watched in detail, but intermittent observation confirmed that the appropriate moults had occurred. Experiments a) and b), therefore, lead to the conclusion that the excretory system is not essential for moulting.

DISCUSSION

Our results indicate that the seam (the median sector of the lateral hypodermis) is necessary for the formation of alae in *C. elegans*. During deposition of the adult cuticle, the surface of the seam forms three ridges which in turn appear to secrete the three alae (Fig. 2B). In dauer formation a thick zone of specialised lateral cuticle is laid down by an enlarged seam before L2 lethargus, and the alae are formed during the subsequent shrinkage of the larva.

When new cuticle, either with or without alae, is being deposited, Golgi bodies are seen in the hypodermis. They are particularly prominent in the seam (Fig. 1, 2C); indeed, it is possible that the few small Golgi bodies seen elsewhere are qualitatively different from those in the seam. Bonner and Weinstein (1972) have demonstrated similar Golgi activity, in the seam of *Nippostrongylus brasiliensis*. Presumably the presence of the Golgi bodies means that the lateral cuticle is specialised in some way. Now, the nematode does not readily bend its body other than dorso-ventrally; in flipping, for example, it is clearly bistable and never remains in intermediate positions. This behaviour suggests that either the lateral hypodermis or the lateral cuticle is inextensible. Perhaps the function of the Golgi bodies is the production of stiff cuticle. This hypothesis is made less attractive by the observation that, in dauer formation, there is little Golgi activity in the seam just before the time of apparent lateral stiffening. However, there is Golgi activity prior to the L2 lethargus, when most of the dauer cuticle is laid down.

The seam is also required for the diametric shrinkage of maturing dauer larvae, since in defective larvae regions lacking the seam do not shrink (Fig. 12). The shrinkage would seem necessarily to involve loss of water through the cuticle, but it seems unlikely that secretion of fluid is the driving force because the effect would then be expected to spread to regions without seam cells. Possibly the specialised lateral cuticle is circumferentially contractile.

Whatever the precise functions of the seam and its Golgi apparatus may be, they do not seem to be essential for survival and growth, since E 1348 and certain other mutants can reach adult size in spite of impairment or total loss of the seam during development.

The seam cells, in addition to playing such a specialised role in moulting, are blast cells: their progeny enlarge the hypodermis and also form the postdeirids and the rays of the male tail (Sulston and Horvitz, 1977). This generative function suggests that they, rather than the syncytia, should be regarded as the more primitive part of the hypodermis.

It has been suggested (e.g. Bird, 1971) that during moulting in some nematodes the excretory duct releases fluid which weakens the old cuticle. In *Mononchus aquaticus* a blister forms at the excretory duct during moulting (Grootaert and Maertens, 1976). *C. elegans* appears to be different. The loosening of the old cuticle clearly begins at the tip of the head rather than at the excretory duct, and the number of refractile granules in the excretory glands (a likely source of moulting fluid) is not cyclic but increases steadily during development. Furthermore, destruction of the excretory glands or of the entire excretory system does not prevent moulting; this is true even of the dauer larva, in which the consistent splitting of the old cuticle close to the excretory duct might suggest local weakening. Thus, the excretory apparatus is not essential for moulting in this nematode, although its synergistic involvement cannot be ruled out. On the other hand, the gl pharyngeal glands are maximally active just before ecdysis (Fig. 6) and hatching. We propose that their secretions soften and loosen the pharyngeal lining, the cuticle around the head, and perhaps the egg shell. It would be interesting to know whether the pharyngeal glands also take part in ecdysis in a nematode such as *Nippostrongylus brasiliensis*, where their digestive function is clearly apparent (Lee, 1968). Bird (1967) has observed pharyngeal gland activity prior to hatching in *Meloidogyne javanica*.

We are very grateful to Nichol Thomson for instruction in and help with serial sectioning, electron microscopy and to Donna Albertson, Bob Horvitz, Dick McIntosh and John White for advice and discussion. A fellowship to R.N.S. from the Nuffield Foundation, London, is gratefully acknowledged.

ZUSAMMENFASSUNG

Einige Beobachtungen über die Häutung beim Nematoden Caenorhabditis elegans

Die Häutung von *Caenorhabditis elegans* wurde an Tieren ausgesuchter Altersstufen mit Hilfe des Interferenzkontrastmikroskopes nach Nomarski und des Elektronenmikroskopes beobachtet. Dabei wurden die Wildtyp-Zellteilungsmutanten und Tiere, in denen Zellen durch einen Laserstrahl abgetrennt worden waren, untersucht. Die median-laterale Hypodermis oder „Saum" ist für die Bildung der Alae und für die Reifung der Dauerlarven erforderlich. Während der Ablagerung der Cuticula sind große Golgikörper in den Zellen des Saumes sichtbar. Das Excretionssystem is für die Häutung unwichtig.

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Accepted for publication: June 6, 1977.