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Freeze-fracture characterization of the cuticle of adult and dauer forms of *Caenorhabditis elegans*

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Abstract At the ultrastructural level, a trilaminate structure, designated as the epicuticle, is always present on the outermost surface of the nematode *Caenorhabditis elegans*. The freeze-fracture technique revealed the existence of two fracture faces: an inner face that showed homogeneously distributed particles and an outer fracture face that was almost completely smooth in adult nematodes but appeared delicately granular in the dauer forms.

Introduction

The epicuticle of nematodes has been the subject of several recent investigations. It is a complex structure that encloses the nematodes except for small openings. Its structure varies between different species and even between different stages of the same species (for reviews see Proudfoot et al. 1991; Selkirk 1991).

Transmission electron microscopy of thin sections of several nematodes has shown that the epicuticle presents a trilaminate pattern resembling a membrane structure (Himmelhock et al. 1977; Bird 1985; Peixoto and De Souza 1992). However, previous freeze-fracture studies of microfilariae of *Onchocerca volvulus* (Martinez-Palomo 1978) and of several stages of *Trichinella spiralis* (Lee et al. 1984, 1986) have failed to reveal intramembranous particles. More recently, however, randomly distributed particles as well as an organized array of particles were seen in the epicuticle of microfilariae of *Wuchereria bancrofti* (De Souza et al. 1993). Therefore, we decided to extend these studies to other nematodes. In the present study, we analysed the cuticle of the free-living nematode *Caenorhabditis elegans*, which takes an alternative developmental pathway in response to overcrowding and limited food at the L2/L3 molt, producing a specialized L3 stage, namely, the dauer larva, that is resistant to desiccation.

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Materials and methods

Nematode strains and culture

The wild-type Bristol strain of *Caenorhabditis elegans* was provided by the Caenorhabditis Genetics Center. The other strain, designated as daf-2, constitutively forms dauer larvae at 25° C. Both strains were grown at 15° C on 100-mm NG agar plates (Brenner 1974) containing 0.5 ml of Roitman's medium (Roitman et al. 1972) using the OP50 strain of *Escherichia coli* as a food source.

Isolation of dauer larval stages

Dauer larvae were purified from other forms by incubation of a nematode suspension with 1% sodium dodecyl sulfate (SDS) for 2 h and centrifugation through 50% ice-cooled Histopaque (Cox et al. 1981).

Light microscopy

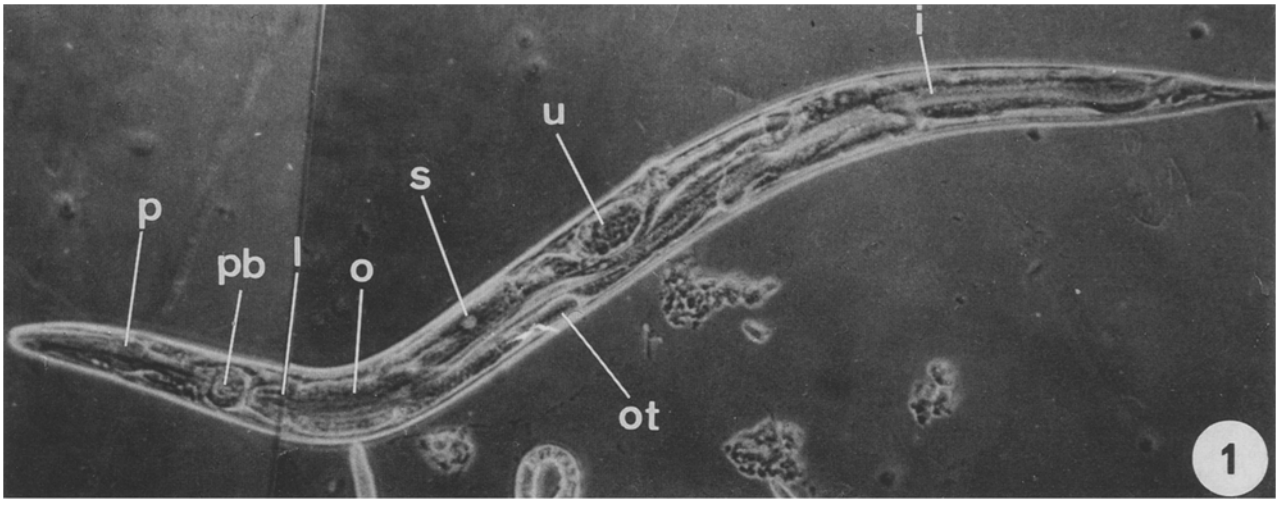
The nematodes were fixed for 2 h at room temperature in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer and were mounted for light microscopy in a Zeiss Universal Microscope using phase contrast.

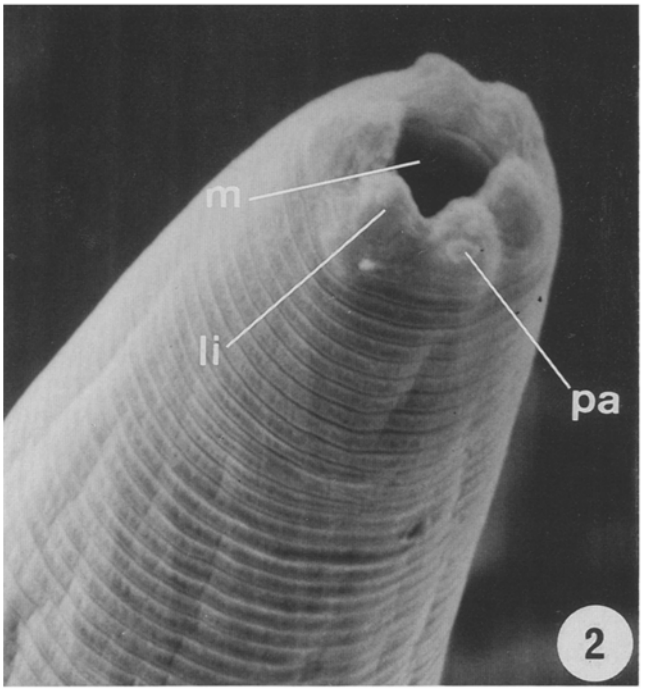
Scanning electron microscopy

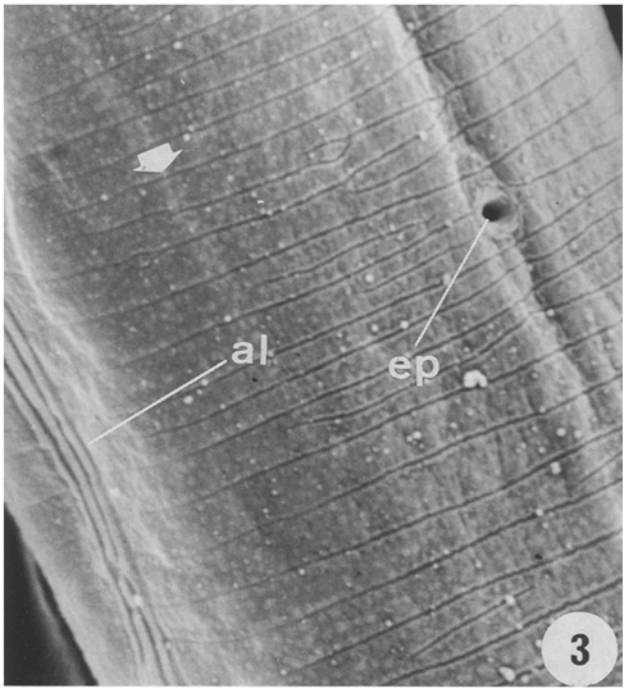
Nematodes were fixed in 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2) for 2 h at room temperature. Then, they were washed twice in the same buffer and post-fixed in 1% buffered osmium tetroxide for 1 h at room temperature. All nematodes were dehydrated in ethanol, washed several times in 100% ethanol, critical-point-dried in CO_2, mounted on stubs, coated with gold and examined in a Jeol 25SII scanning electron microscope.

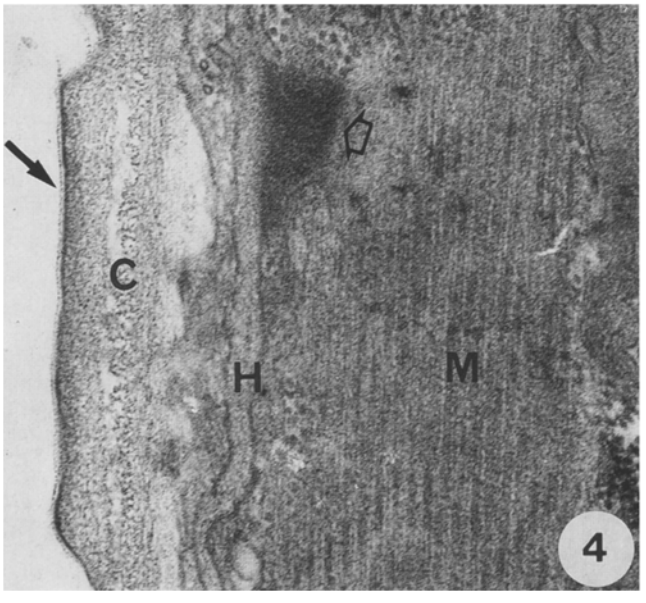
Transmission electron microscopy

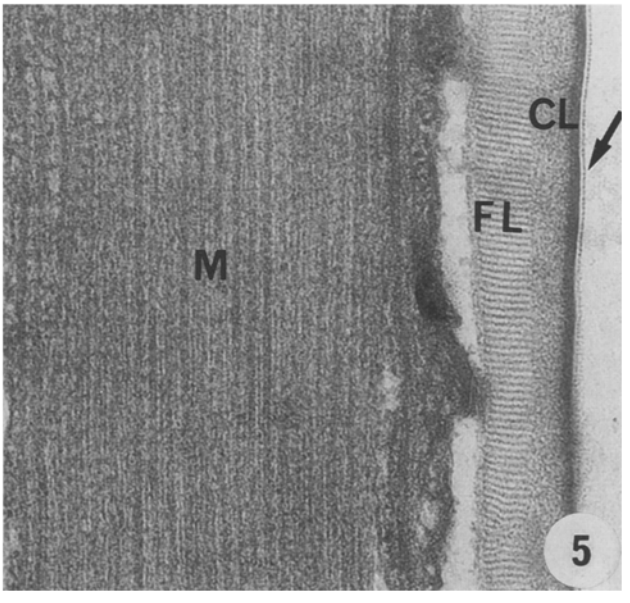
The nematodes were fixed for 2 h at room temperature or overnight at 4° C in 2.5% glutaraldehyde in 0.1 *M* phosphate











Figs. 1–5

buffer (pH 7.2). After fixation the nematodes were washed twice in the same buffer and once in cacodylate buffer (pH 7.2), after which they were post-fixed in a solution containing 1% osmium tetroxide, 2 m*M* $CaCl_2$ and 0.8% potassium ferricyanide in 0.1 *M* cacodylate buffer (pH 7.2). They were then dehydrated in acetone and embeed in Spurr's medium (Spurr 1969). Thin sections were stained with uranyl acetate and lead citrate.

Freeze-fracture study

The nematodes were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.2) for 2 h at room temperature. After fixation, they were washed twice in the same buffer and then exposed to ascending concentrations of glycerol in 0.1 *M* cacodylate buffer until a final concentration of 30% (v/v) glycerol was attained (after 30 min). The nematodes remained in 30% glycerol in cacodylate buffer for 2 h. Specimens were mounted on Balzer's support disks and then rapidly frozen in liquid Freon 22 cooled by liquid nitrogen. Freeze-fracture was carried out at −115° C in a Balzer's apparatus. The specimens were shadowed with platinum/carbon at 2×10^{-6} Torr immediately after fracture. Replicas were recovered in distilled water, cleaned with sulphuric acid and sodium hypochlorite, mounted on 200-mesh grids and observed in a Zeiss 900 transmission electron microscope.

Results and discussion

Figure 1 shows the general morphology of an adult form of *Caenorhabditis elegans* as seen by phase contrast. The bulbar pharynx, tubular intestine and symmetrically bilobed gonad are evident. Scanning electron microscopy of the anterior region showed a large triradiate mouth surrounded by six lips, each showing labial papillae (Fig. 2). The excretory pore appeared as a small, rounded structure about 1.0 μm wide (Fig. 3).

Examination of thin sections of adult nematodes showed six layers, as previously described (Peixoto and De Souza 1992): a trilaminate epicuticle, an external cortical layer, an internal cortical layer, an intermediate layer, a fibrous layer and a basal layer (Fig. 4). Thin sections of a dauer form showed the following layers: epicuticle, external cortical, internal cortical, intermediate, fibrous composed of thin and thick fibers, and basal (Fig. 5).

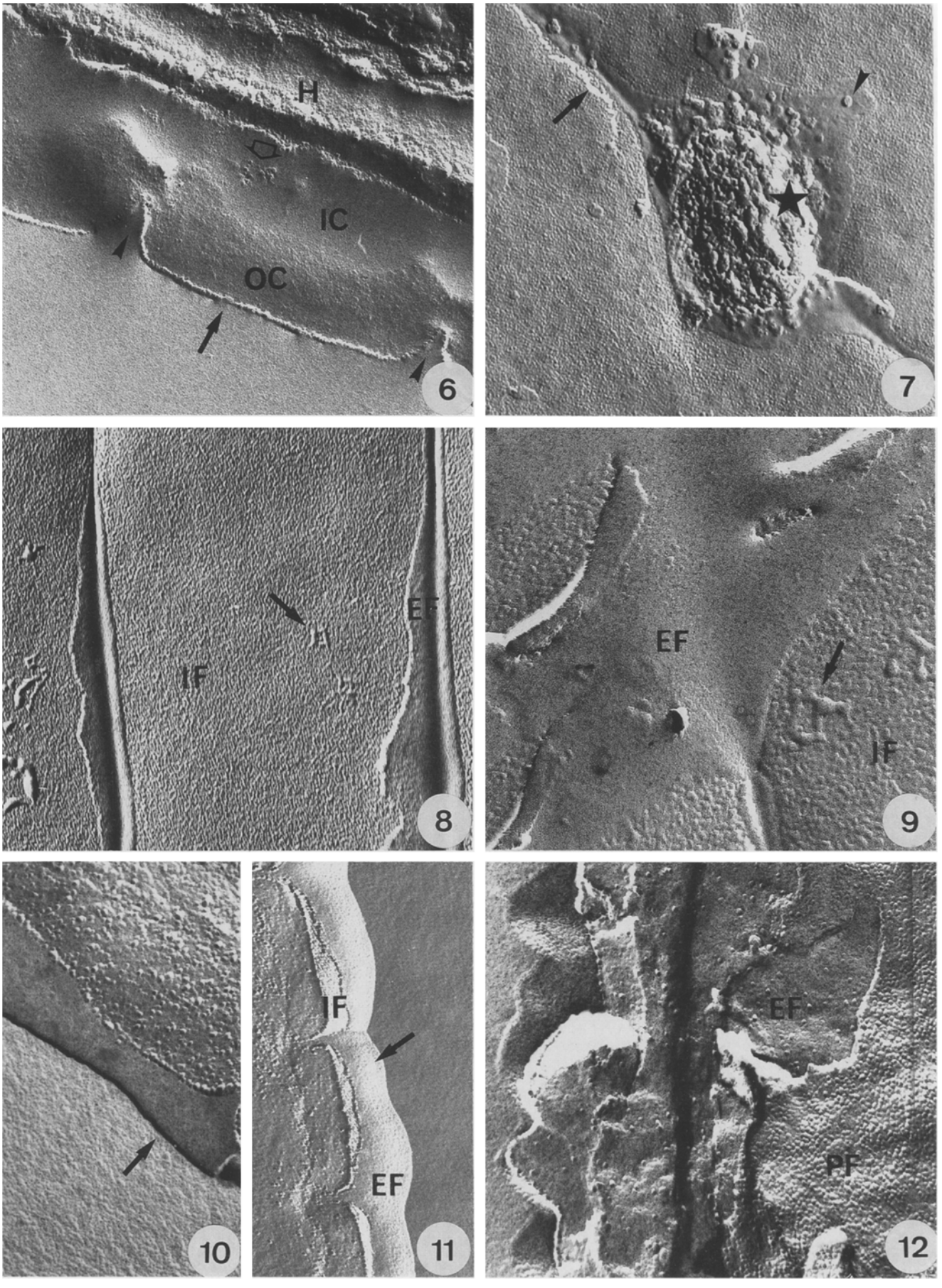
Fig. 1 An adult nematode observed by phase-contrast light microscopy. Structures such as the procorpus (*p*), posterior bulb (*pb*), intestine (*i*), loop (*l*), oviduct (*ov*), spermatheca (*s*) and uterus containing an egg (*u*) are indicated. × 175, **Figs. 2, 3** Scanning electron micrographs of *Caenorhabditis elegans*. **Fig. 2** En face view of the triradiate mouth surrounded by six lips (*li*), each bearing sensorial papillae (*pa*). × 2800. **Fig. 3** Ventral view of an adult nematode near the pharynx. Annuli are visible at regular intervals (*arrow*) interrupted by trilobed ala (*al*). The excretory pore (*ep*) is also indicated. × 4300. **Fig. 4** Longitudinal thin section of an adult nematode, showing the epicuticle (*arrow*), cuticle (*C*), hypodermis (*H*), dense bodies (*open arrow*) and muscle cell (*M*). × 35000. **Fig. 5** Longitudinal thin section through a dauer larva, showing the characteristic fibrous layer striation (*FL*). The epicuticle (*arrow*), cortical layers (*CL*), and muscle cell (*M*) can be seen. × 45000

The trilaminate appearance of the epicuticle was evident in both adult and dauer forms. Previous studies have shown that there is considerable heterogeneity in cuticle structure between different species and even between different stages of the same species. However, a trilaminate structure is always present as the outermost layer of the nematode's cuticle (Bird 1980; Wright 1987). In some cases, as in microfilariae of *Wuchereria bancrofti*, this structure is more complex, showing a heptalaminar organization (De Souza et al. 1989).

Several experiments were done to obtain freeze-fracture replicas that showed the epicuticle. In most cases, relatively small areas of epicuticle were exposed. In other situations, however, fracture faces of the epicuticle were clearly exposed, allowing their examination. Cross fractures clearly revealed the periodic array of annulations (Fig. 6). The epicuticle appeared as an almost continuous line composed of tightly arranged particles (Fig. 6). No detail could be seen when the fracture plane exposed the cuticle. However, it was clear that the outer half portion of the cuticle presented a more granular pattern as compared with the inner portion, where aggregated particles were occasionally seen (Fig. 6). Immediately below the cuticle the hypoderm was observed. Occasionally, plasma-membrane fracture faces of the hypodermal cell were exposed. The protoplasmic fracture face showed a high density of typical intramembranous particles with a mean diameter of 16.5 nm (Fig. 12). Very few particles with the same diameter were seen on the extracellular fracture face of the hypodermal cell membrane (Fig. 12).

Longitudinal fractures through the epicuticle revealed the presence of two fracture faces localised at two distinct levels. These faces were seen even at the region of the excretory pore (Fig. 7). The outer layer was almost completely smooth, although it occasionally presented globous structures with a mean diameter of 77.0 nm. These structures were very frequently seen in the region of the excretory pore (Fig. 7). The inner fracture face presented a large number (about 805/μm^2) of homogeneously distributed particles with a mean diameter of 12.5 nm (Figs. 7–9). At some regions, rounded structures resembling a rosette, with an 84-nm-thick wall and an inner diameter of about 60.0 nm, were observed. This structure seemed to be formed by closely associated particles. A similar structure has previously been observed in the epicuticle of microfilariae of *W. bancrofti* (De Souza et al. 1993) Longitudinal fractures through the dauer epicuticle also revealed two fracture faces: a particulated inner face and an outer fracture face not as smooth as that observed on the adult epicuticle, with a delicate granular appearance (Fig. 11).

Very few studies have aressed the characterization of the epicuticle of nematodes. Previous freeze-fracture studies have revealed the presence of particles on the epicuticle of *Meloidogyne* (Bird 1984) but not in *Trichinella spiralis* (Lee et al. 1984) or *Onchocerca volvulus* (Martinez-Palomo 1978). Our previous observations in microfilariae of *W. bancrofti* clearly showed



Figs. 6–12

the presence of two fracture faces with a distinct density of particles (De Souza et al. 1993). The present study extends those observations to the free-living nematode *C. elegans*. Particles such as those seen in freeze-fracture replicas have been shown to correspond to membrane-associated proteins at least partially embeed in a lipid bilayer (for a review see Verkleij and Ververgaert 1978). The presence of proteins and glycoproteins on the outer face of nematodes, presumably in the epicuticle, has been demonstrated by extrinsic radio-iodination experiments (for a review see Selkirk 1991). Biochemical experiments as well as the use of fluorescent lipid probes have suggested the presence of lipids and glycolipids in the nematode surface (Kennedy et al. 1987; Scott et al. 1988; Proudfoot et al. 1991).

Taken together, these observations suggest that the epicuticle may constitute a special type of membrane organization. Our attempts to analyse its organization further through the use of drugs such as filipin (for a review see Severs and Hobeneck 1983) and polimixin B (Sixl and Galla 1981), which interfere with the array of membrane lipids in other cells, did not provide aitional significant information (Peixoto and De Souza, unpublished observations). Certainly, many more morphology and biochemistry studies in different species are necessary to provide a clear picture of the topochemical organization of the cuticle of nematodes.

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Fig. 6 Freeze-fracture image of the cuticle, showing the inner (*IC*) and outer (*OC*) portions. The arrangement of homogeneous particles bearing the epicuticle is indicated (*arrow*). Annuli (*arrowheads*), the hypodermis (*H*), and the muscle cell are shown. ×4000. **Fig. 7** Freeze-fracture image showing the excretory pore (*star*). Annuli are indicated (*arrow*). ×29000. **Figs. 8, 9** Freeze-fracture images of the epicuticle of an adult nematode, showing the inner (*IF*) and the external face (*EF*). Circular arrangements of particles on the inner face are evident (*arrow*). **Fig. 8** ×22000; **Fig. 9** ×67500. **Fig. 10** Freeze-fracture image of an adult cuticle. The smooth external face is indicated (*arrow*). ×35000. **Fig. 11** Freeze-fracture image of a dauer-larva cuticle. *IF*, Inner face; *EF*, external face. Homogeneously distributed particles are visible on the external face (*arrow*). ×35000. **Fig. 12** Freeze-fracture image of the adult hypodermis. Densely distributed particles are evident on the protoplasmic face (*PF*) of the plasma membrane of a hypodermal cell. A few homogeneously distributed particles are visible on the extracellular face (*EF*). ×40000

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