THE CUTICLE OF THE PARASITIC NEMATODE, *Nematospiroides dubius*

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There once was a worm called *N. dubius*,
Whose habits were really quite curious.
He shunned the fresh air,
And lived without care
In the gut of the mouse *Mus musculus*.

(Hurley, 1984).

CONTENTS

SUMMARY

The cuticle of nematodes is of interest because it forms the boundary between the animal and its environment. In this thesis, the cuticle of the parasitic nematode, Nematospiroides dubius, was examined with particular attention being focused on the epicuticle.

The fourth-stage cuticle of N. dubius, at least, is an extracellular structure which probably forms by self-assembly from molecules secreted by the epidermis. Because cuticle formation is a fundamental process, it is assumed that the cuticle of each stage forms in a similar way.

The structure of the cuticle of N. dubius, which is highly variable, appears to be related to the environment in which the worm lives and, in particular, to parasitism. The cuticle of the parasitic stages, that is, the fourth-stage and adult worms, are similar but differ markedly from those of the first three stages. The cuticle of the free-living stages exhibit longitudinal specializations, the lateral alae, which run along each side of the worm. In contrast, the entire cuticle of the parasitic forms was thrown into a series of longitudinal ridges. Moreover, a fuzzy electron-dense coat could be detected only on the surface of the parasitic worms.

The third-stage cuticle had some unusual properties. Unlike the other stages, the epicuticle of third-stage larvae was multilaminate when viewed in transverse section, and did not exhibit a net negative charge. Furthermore, substantial ultrastructural changes in the body wall of third-stage larvae occurred during the transition to parasitism, and these appeared to be related to behavioural changes.

The composition of the epicuticle of N. dubius varies amongst stages. The epicuticle of adult worms, at least, appears to depend on disulphide bonds because it was found to be susceptible to reduction. In addition, some molecular interactions within the epicuticle might be hydrophobic, because the epicuticle was affected by a detergent.

The adult epicuticle split when freeze-fractured to reveal two fracture faces. This result, together with the effect that a variety of stains and organic solvents had on the surface of the epicuticle, suggests that a major component of the epicuticle might consist of a bilayer of lipid in some form, possibly lipopolysaccharide. Protein and/or glycoprotein also probably form an important part of the adult epicuticle.

The third-stage cuticle differed from that of adult worms. The epicuticle did not split when freeze-fractured, nor did it react with many of the stains used to highlight the surface of adult worms.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text. If accepted for the award of the degree, the thesis is available for photocopying and loan.

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THIS THESIS IS DEDICATED TO MY PARENTS

WHO NEVER ONCE SAID THAT I HAD TO DO IT.

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CHAPTER 1

INTRODUCTION

1.1 A GENERAL INTRODUCTION TO THE THESIS

The cuticle of nematodes forms the interface between animal and environment. As such, it must play an important role in the relationship between the organism and its habitat. The cuticle represents the first barrier for substances entering the animal, and the last barrier for substances leaving it.

Molecules making up the cuticle appear similar in origin and composition to those found at the surface of a variety of organisms. The cuticle of arthropods, the shell of molluscs, the outer wall of bacteria and plant cells, and the extracellular matrix of multicellular organisms, for example, share some fundamental properties (Hay, 1981; Larman, 1984; Locke, 1982). The precursors of these structures all appear to be secreted locally by cells and then, in part, self-assemble (Linsenmayer, 1981; Trelstad & Silver, 1981). Some aspects of the assembly, such as the timing and location, may be regulated by the underlying cells (Trelstad & Silver, 1981). In general, extracellular structures are composed of collagens, proteoglycans and/or structural glycoproteins (Hay, 1981; Hewitt & Martin, 1984).

Before discussing the structure of the cuticle of nematodes in detail, it is appropriate to consider the function of some of these related surface structures.

1.1.1 The metazoan cell, and the extracellular matrix

The surface of cells making up most multicellular organisms is in

contact with an intricate meshwork of interacting macromolecules known as the extracellular matrix (Hay, 1981). Together the cell surface and the extracellular matrix all but determine the size and shape of these organisms. Not only do components of the extracellular matrix serve as biological glue, but they also form such specialized structures as cartilage, tendons, ligaments, skin, basal laminae and, with mineralization, bones and teeth (Hascall & Hascall, 1981; Linsenmayer, 1981).

As well as maintaining the skeletal structure of multicellular organisms, components of the extracellular matrix and cell surface are thought to govern interactions between cells. Such interactions are of fundamental importance in embryogenesis (Frazier & Glaser, 1979; Glaser, 1980). The extracellular matrix is thought to influence the movement, proliferation, recognition and adhesion of cells via specific receptors located within the "glycocalyx", that is, the carbohydrates present on the outer surface of cells (Toole, 1981). In turn, these receptors are thought to specifically interact with such agents as hormones and antibodies (Frazier & Glaser, 1979; Goodenough & Adair, 1980; Hughes, 1975; Nicolson, Poste & Ji, 1977; Yamada, Olden & Hahn, 1980). Cell surface receptors also recognize cells belonging to the body as "self", while those foreign to the body are identified as "non-self" and destroyed by the immune system.

Less specific functions have also been suggested for the glycocalyx. Molecules on the surface of cells are thought to restrict the diffusion and transport of solutes, and in other circumstances, to lubricate and protect cells (Bennet, 1963; Ito, 1969; Luft, 1976; Kornfeld & Kornfeld, 1980).

Thus the extracellular matrix and the cell surface together govern

a number of aspects of the structure and growth of multicellular organisms.

1.1.2 Procaryotes and invertebrates

The surface region of the lower organisms, which is often composed of an extracellular matrix of molecules, may fulfil a variety of functions. As well as regulating the inflow of required molecules and outflow of less desirable ones, the surface provides protection and/or support and tensile strength (Linsenmayer, 1981). Bacteria, for example, are surrounded by a protective extracellular wall which not only protects the plasma membrane from lysis, but also increases the area available for metabolic activity by enclosing the so-called periplasmic space around the cell membrane (Inouye, 1979). Likewise, the precursor elements for the cuticle of insects and for the shell of molluscs are secreted outside the plasma membrane, where they protect and support the underlying tissue (Locke, 1966; Locke & Huie, 1979; Saleuddin, 1975).

But not all of the functions attributed to the surface region of these organisms involve support or protection. Adhesion of Escherichia coli to human mucosal cells, for example, is dependent on a lectin present on the surface of the bacterium (Ofek, Mirelman & Sharon, 1977). In addition, the surface chemistry of the feeding structures of filter feeders, such as Daphnia magna, in part determines the size of the particulates the organism can catch (Gerritsen & Porter, 1982).

Further interesting examples of surface interactions at work are provided by the fungi, Arthrobotrys oligospora and Dactylaria candida, which trap and consume small nematodes (Nordbring-Hertz & Mattiasson, 1979; Nordbring-Hertz, Friman & Mattiasson, 1982). The

recognition mechanism is thought to involve a lectin on the surface of the fungus which identifies and binds to a sugar on the surface of the nematode. Similarly a sugar on the surface of the endoparasitic fungus, Meria coniospora, is thought to bind specifically to sialic acid on the surface of Panagrellus redivivus during infection (Jansson & Mattiasson, 1983; 1984).

The surface of many of the lower organisms, therefore, is composed of an extracellular matrix of macromolecules which have complex and varied functions. When this surface region is viewed in transverse section using transmission electron microscopy, it often appears trilaminate. Locke (1982) proposed that the term "envelope" be used to describe these laminate surface structures which resemble plasma membranes in appearance and dimensions. Unlike plasma membranes, however, envelopes always self-assemble, in part at least, from precursor elements outside the plasma membrane.

1.1.3 The surface of parasites, a special case

The surface of parasitic organisms is of particular importance because it forms the interface between the parasite and its host (Smyth, 1976). Not only is there an interchange of materials of physiological importance across this region, but antigenic components on the surface of an invading parasite are recognized as "non-self" by host cells which produce antibodies against them (Bloom, 1979; Ogilvie, Philipp, Jungery, Maizels, Worms & Parkhouse, 1980; Smithers & Terry, 1976). Some parasites elude the immune system of their host by varying the antigenic components on their surface, or by acquiring host antigens (Bloom, 1979; Cross, 1978; McClaren & Terry, 1982). Others, such as the fluke Fasciola hepatica, respond to the hostile environment within the host by undergoing a rapid turnover of surface components

which prevents host cells from adhering to the surface of the worm (Hanna, 1980).

Thus the surface of parasites is important, not simply because it is the boundary of the organism, but because it may form the target of the immune system of the host.

1.1.4 The parasitic nematode, NEMATOSPIROIDES DUBIUS

The focus of this thesis is the surface of the parasitic nematode, Nematospiroides dubius. The first two juvenile stages of this nematode are free-living but the third-stage larva must make the transition to parasitism. Fourth-stage larvae and adult worms parasitize the small intestine of mice.

The surface of nematodes is composed of a laminate structure, the epicuticle, when viewed in transverse section (Bird, 1971; 1980; 1984; Lee, 1977; Lee & Atkinson, 1977; Nicholas, 1984). There is strong evidence to suppose that the epicuticle bounding the nematode cuticle is extracellular and is therefore, according to Locke (1982), an "envelope" (Bonner & Weinstein, 1972a; Martinez-Palomo, 1978); however, some doubt remains (Lee, 1970; Bird, 1980; 1984). If the epicuticle forms outside the epidermal membrane then it may share some biochemical properties with other envelopes. Alternatively, if the epicuticle forms within the epidermal membrane, as has been suggested, then the surface of the nematode would be a plasma membrane and so might resemble the surface of cells.

The major thrust of this thesis is to discover the origin and composition of the epicuticle of N. dubius. Before examining the results of studies into the nature of the epicuticle of N. dubius, however, the literature relating to the cuticle of nematodes in general will be reviewed.

In this review, particular attention shall be paid to the nature of the epicuticle of nematodes because it is the surface region of N. dubius that forms the focus of this study. The ultrastructural studies of the inner three zones have been extensively reviewed and little more can be added at this stage (Bird, 1971; 1976; 1980; 1984; Bird & Bird, 1969; Lee, 1977; Lee & Atkinson, 1977). Ultrastructural and biochemical studies shall be examined in an effort to shed some light on the composition of the surface of nematodes.

1.2 REVIEW OF LITERATURE ON THE CUTICLE OF NEMATODES

Despite a great deal of work done on the cuticle of nematodes, many questions about both structure and function remain unanswered. Much work has centred on the different layers making up the cuticle but the ultrastructural details of the surface have been neglected (Bird, 1971; Inglis, 1964). Some generalizations about the nematode cuticle may be made, however, bearing in mind that generalizations hide a wealth of interesting, and perhaps even important, exceptions.

There seems general agreement that the nematode cuticle consists of, or has evolved from, a four-layered structure which lies above the cellular dermal region* (Bird, 1980; McLaren, 1972). In addition, the

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*In this thesis, the term "epidermis" will be used to describe the cellular region beneath the cuticle in nematodes, after Lee (1977). The term "hypodermis", which is generally employed for nematodes, will not be used because it also refers to subcutaneous tissue in vertebrates.

ultrastructure of the cuticle appears to be related to the environment in which the worms live, and the structure of the cuticle can change without a new cuticle being synthesized. These three generalizations will now be considered.

The cuticle is stratified

Four different layers or zones can be located in the cuticle of nematodes, but each zone need not be present in all taxa (Bird, 1971; 1980; Lee, 1977; Lee & Atkinson, 1977; Maggenti, 1979). The identity of the inner three zones, that is the cortical, median and basal zones, is firmly established (Fig. 1.1; Bird, 1971; 1980; Lee, 1977), but some confusion surrounds the definition of the outermost region, the epicuticle.

On the one hand, the epicuticle has been defined as the triple-layered structure common to the surface of all nematodes (Bird, 1980). However, although the epicuticle often appears trilaminate in transverse section, it may be multilaminate (Dick & Wright, 1973; Jenkins, 1969; Smith, 1970). And in some nematodes a well-defined electron-dense coat of filaments may extend from the surface (Hamada & Wertheim, 1978; Lee, 1970; Morseth & Soulsby, 1969). Because the epicuticle of nematodes varies in ultrastructure, it seems appropriate to consider the epicuticle as a region or zone, rather than a triple-layered structure.

Another reason for referring to the epicuticle as a zone lies in the origin of the term. Bird (1971) noted that the surface of the nematode cuticle consisted of an outer electron-dense line of 7 nm, a middle electron-lucent line of 10 to 20 nm and an inner electron-dense line, thicker than the first (Fig. 1.2a). He went on to state that the outer electron-dense line could be resolved as a triple-layered structure which resembled a plasma membrane in appearance (Fig. 1.2a).

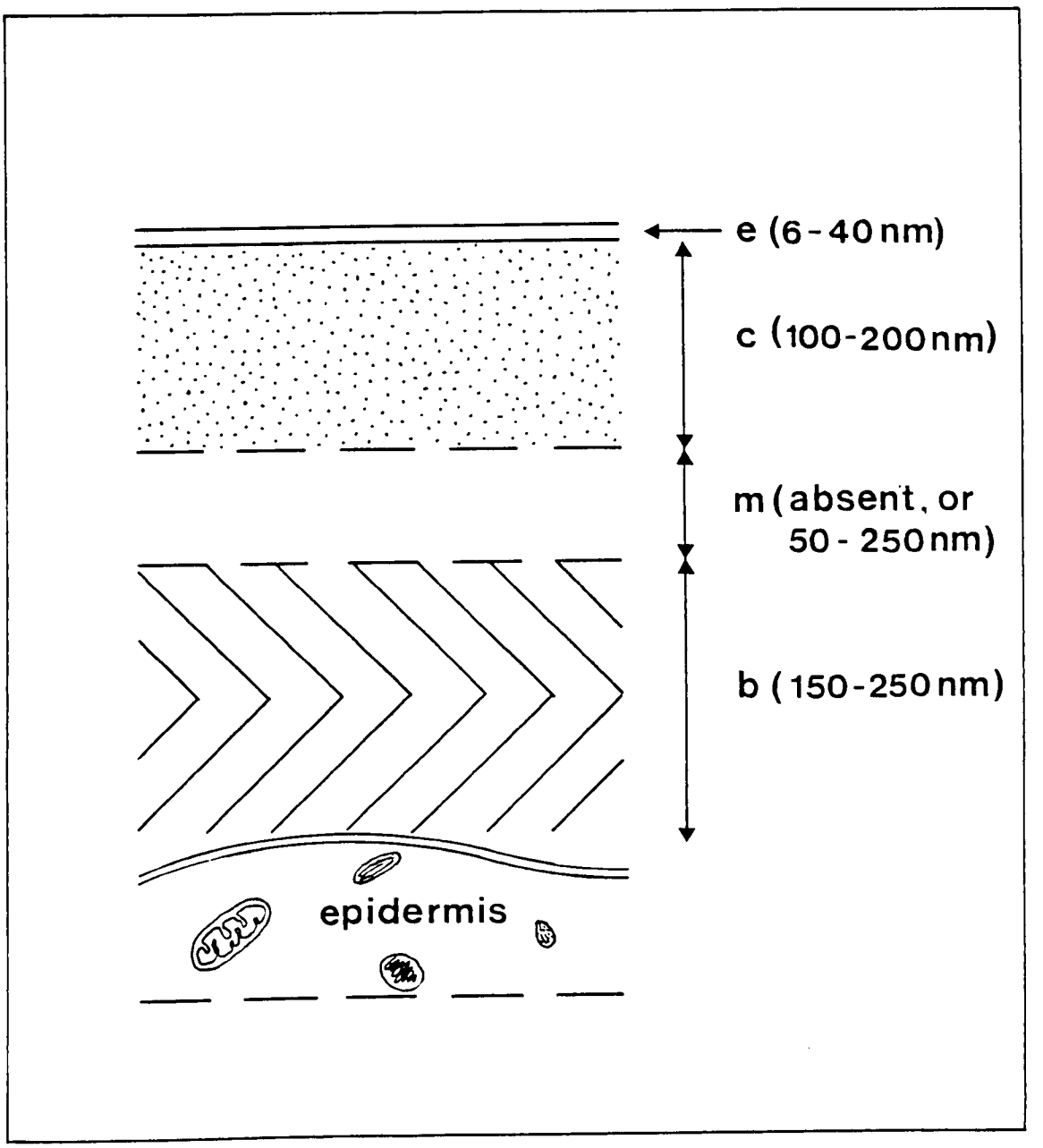


Fig. 1.1: Diagram of a generalized nematode cuticle showing the positions of the epicuticle (e), and the cortical (c), median (m), and basal (b) zones, relative to the underlying epidermis (after Bird, 1980).

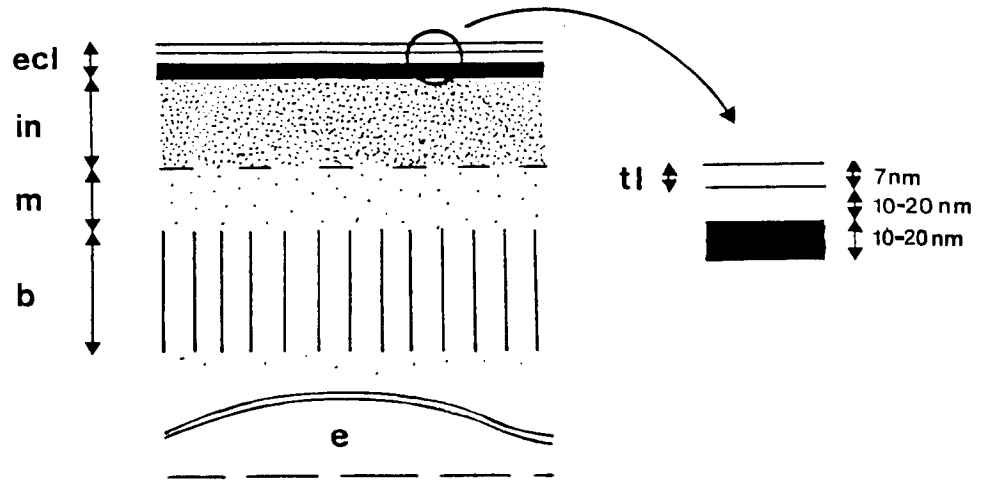


Fig. 1.2 a

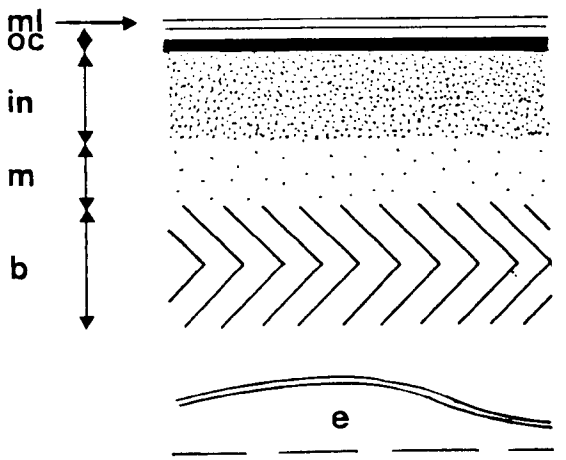


Fig. 1.2 b

Fig. 1.2: Diagrams showing the confusion in the terminology of the outermost region of the nematode cuticle (Fig. 1.2a, after Bird, 1971; Fig. 1.2b, after Lee, 1977). basal zone, b; external cortical layer, ecl; inner cortical zone, ic; median zone, m; outer cortex, oc; membrane-like layer, ml; triple-layered membrane, tl.

But as mentioned earlier, Bird (1980) later defined the epicuticle as "a triple-layered structure common to the surface of all nematodes" (Fig. 1.1). If one assumes that the outermost triple-layered structure is the epicuticle, as one would do following Bird's (1980) definition, then the inner dense line of 10 to 20 nm (Fig. 1.2a) has not been included in the definition of "epicuticle". Lee (1977), on the other hand, recognized the outermost triple-layered structure but retained the term "outer cortex" to describe the inner dense layer (Fig. 1.2b). In this thesis the epicuticle will be thought of as the laminate zone or region that bounds the nematode cuticle.

Beneath the epicuticle lies the cortical zone which is usually made up of single fibrils and measures between 100 and 200 nm in width (Fig. 1.1; Lee, 1977). When stained with lead nitrate and uranyl acetate, this region appears electron-dense but amorphous.

The third region moving inwards is the median zone which varies greatly between different species of nematode. When present it may measure between 50 to 200 nm in thickness (Fig. 1.1, Bird, 1980). The cuticle of adult Nippostrongylus brasiliensis, for example, has a wide median zone which contains a complex array of struts (Lee, 1965; Jamuar, 1966). In contrast, the median zone is absent altogether in the pathogenic female parasite of the bush-fly, Heterotylenchus sp. (Nicholas, 1972).

The basal zone forms the innermost region of the cuticle and is separated from the underlying epidermis by the epidermal plasma membrane (Fig. 1.1; Sommerville, 1982). This zone is thought to be the major skeletal region of the cuticle (Wisse & Daems, 1968; Lee, 1977) and more often than not, consists of a number of fibre layers, as for example in Ascaris lumbricoides and Trichuris suis (Jenkins, 1969; O'Grady, 1983; Watson, 1965).

The basal zone of the cuticle of many free-living stages of nematode, on the other hand, contains striated material of regular periodicity. The cuticle of the dauer larva of Caenorhabditis elegans has such a layer, as do the cuticles of the infective-stages of Haemonchus placei, N. brasiliensis and Meloidogyne javanica, to name just a few (Bird, 1968; Cox, Staprans & Edgar, 1981a; Lee, 1966; Popham & Webster, 1978; Smith, 1970). The composition of the striated layer has captured the imagination of a number of workers. Popham & Webster (1978), for example, suggested the striated material represents the cut surfaces of blocks of polymerized protein separated by sheets of osmiophilic lipoprotein.

Dividing the cuticle into these four zones is convenient for the observer. Of course, the zones probably do not function independently; rather it seems likely that the cuticle operates as a functional unit.

Cuticle structure and the environment

A second generalization to note about the cuticle of nematodes is that there is a tendency for the structure to be related to the environment in which the worm is found (Bird, 1971; Davey, 1982; Philipp & Rumjaneck, 1984).

In parasitic nematodes, which pass through several types of environment, the cuticle of the different life stages often vary. For example, the cuticle of larval forms of Phocanema decipiens and A. lumbricoides, which reside in host tissue, differ from that of adults living in the lumen of the intestine (Davey, 1965; Thust, 1966; Watson, 1965). Similarly, the cuticle of the free-living infective-stage of N. brasiliensis is quite distinct from that of the adult worm which lives in the intestine of the host rat (Jamuar, 1966; Lee, 1965; Lee, 1966). In comparison the cuticles of free-living nematodes, such as Panagrellus silusiae and C. elegans, which do not live in a

"discontinuous" environment, show less variation in structure between stages (Cox et al., 1981a, Edgar, Cox, Kusch & Politz, 1982; Samoiloff & Pasternak, 1968).

The cuticle of free-living larvae of parasitic nematodes tends to be more similar to that of other free-living nematodes than to the cuticle of the parasitic adults of the same species. The third-stage cuticles of N. brasiliensis (Lee, 1966), H. placei (Smith, 1970), Heterotylenchus sp. (Nicholas, 1972) and second-stage larvae of M. javanica (Bird, 1968), for example, are similar to the cuticle of adult C. elegans and P. silusiae (Cox, Kusch & Edgar, 1981b; Samoiloff & Pasternak, 1968). But the cuticle of parasitic stages of these species tends to differ from that of the free-living stages.

The cuticle of nematodes that pass through different types of environments, therefore, often vary in structure. On the other hand, there is a tendency for the cuticle of nematodes, which live in a relatively constant environment, to be similar in structure.

Changes in the cuticle between moults

A third point to note about the nematode cuticle is that it can alter between moults. The second-stage cuticle of M. javanica, for example, changes in appearance within two days of the worm infecting a new host (Bird, 1968). More spectacular is the example afforded by A. lumbricoides. As the adult worm grows in length from 20 to 150 mm after the final moult, the cuticle undergoes a proportional increase in thickness (Roberts, 1934; Watson, 1965).

In summary, the cuticle of nematodes forms the boundary between animal and environment and is composed of, or has evolved from, a four-layered structure. The structure of the cuticle, which varies widely, may alter between moults, and there appears to be a relationship

between cuticular structure and the environment in which the worm is found.

1.2.1 The ultrastructure of the epicuticle

As suggested above, the structure of the epicuticle appears to be related to the environment in which the nematode lives. Considering the range of environments in which nematodes live, it is not surprising that the structure of their surfaces vary.

Before going on to consider the relationship between epicuticular structure and environment, it seems appropriate to briefly consider why variations in the structure of the epicuticule have often been overlooked.

In the past there has been a tendency to emphasize the number and structure of the major zones of the cuticle. Consequently the magnification used in some ultrastructural studies was insufficient to resolve the fine structure of the epicuticle (Bird, 1980). For example, in an extensive survey of the cuticle of the genus Heterodera, the ultrastructure of the epicuticle could only be resolved in a few micrographs because the magnification used was, for the most part, insufficient (Shepherd, Clark & Dart, 1972). Other studies have avoided the problem; indeed Raski & Jones (1973) simply presented a diagram of the surface of the nematodes Bunonema richterse and Bunonema reticulatum, maintaining that they did not "definitely see" the outermost osmiophilic layer. Other examples are abundant (Eckert & Schwarz, 1965; Colley, 1970; Himmelhoch, Kisiel, Lavimoniere & Zuckerman, 1973; Kisiel, Himmelhoch & Zuckerman, 1972; Fredericksen & Specian, 1981). In particular, overstaining of sections is thought to obscure some of the fine structure (Bird, 1980).

Despite the technical difficuties, the epicuticle of some nematodes has been clearly shown to be a laminate structure when viewed

in transverse section. Although usually trilaminate, the epicuticle may be multilaminate (Bird, 1980; Lee, 1977; Nicholas, 1984); a point often overlooked (Bird, 1980; 1984; Roggen, Raski & Jones, 1967).

With few exceptions, a multilayered epicuticle has been detected only in parasitic or specialized free-living nematodes. For example, Jenkins (1969) established the presence of several electron-dense lines bounding the cuticle of adult T. suis in some excellent micrographs. The ultrastructural details of the surface of T. suis, however, appear to be a source of confusion to the author. He maintains that T. suis is bounded by a single layered membrane but notes the presence of at least one other layer, and sometimes two, external to the first. Examination of his crisp micrographs reveals that in addition to these four electron-dense lines, the region that Jenkins (1969) terms the outer cortex in the terminology of Lee (1977), is composed of at least two more electron-dense lines.

Similarly the surface of Capillaria hepatica consists of four parallel electron-dense lines (Wright, 1968) while the surface of migrating females of Syphacia obvelata was found to be bounded by a double membrane (Dick & Wright, 1973). In a particularly clear micrograph (Plate 9 in Dick & Wright, 1973), five osmiophilic lines can be discerned at the surface of the worm. Baldwin and Hirschman (1975) also detected five electron-dense lines within the epicuticle of the anterior region of male Meloidogyne incognita but did not comment on the complexity of its structure.

A multilayered epicuticle is not restricted to adult nematodes. The epicuticle of infective-stages of H. placei, M. incognita and Trichostrongylus colubriformis is also multilaminate (Johnson & Nusbaum, 1970; Smith, 1970; Wharton, Perry & Beane, 1981). In addition the surface region of the first-stage larvae of Trichinella spiralis

taken from host muscle cells consists of four electron-dense layers, but it is not known whether the outermost layers are derived from the host or from the parasite (Despommier, 1975).

The nematodes for which a multilayered surface have be detected, therefore, are often parasitic or a specialized free-living stage. Further evidence that the structure of the cuticle of nematodes is related to the environment in which the worms are found comes from a survey of published electron micrographs.

The ultrastructure of the surface region of the cuticle of free-living nematodes appears to generally differ from that of parasitic stages. While the surface of parasitic nematodes tends to be fringed by a well-defined surface coat which is electron-dense, any surface coat on free-living nematodes is difficult to detect when stained with lead citrate and uranyl acetate (Bonner & Weinstein, 1972a; Cox et al., 1981a; 1981b; Hamada & Wertheim, 1978; Lee, 1965; 1966; 1970; Morseth & Soulsby, 1969). For example, while a dense surface coat is present on the cuticle of fourth-stage larvae and adults of N. brasiliensis, no surface coat was described on the cuticle of third-stage larvae of the same species, nor could one be discerned in the micrographs (Lee, 1965; 1966).

In keeping with the observation that the structure of the epicuticle may vary in different environments, it is interesting to note that that the epicuticle of the microfilariae, which occupy unusual habitats, differs from that of the other nematodes. The surface coat of microfilariae does not have the filamentous appearance characteristic of other nematodes, but instead is very dense and is always absent from the annulations (Johnston & Stehbens, 1973; Lehane, 1978; Laurence & Simpson, 1974; McClaren, 1972; Martinez-Palomo, 1978). The material at the outer edge of the epicuticle of Dipetalonema viteae, for example,

appears to be laminate and so bears little semblance to a "fuzzy" surface coat (Plate 2 in Ouaissi, Haque & Capron, 1982).

While ultrastructural differences are particularly apparent between the cuticles of parasitic and free-living nematodes, the surface of some specialized free-living nematodes may also be influenced by the environment. When faced by unfavourable conditions, the parasite Anguina tritici enters an anhydrobiotic state. The outermost osmiophilic layer of the epicuticle appears to be more dense than the inner layer in anhydrobiotic forms, but in hydrated worms the inner line was more dense than the outer one (Bird & Buttrose, 1974). Similarly the epicuticle (called "thin external cortical layer") of the pinewood parasite Bursaphelenchus lignicolus, and that of the free-living C. elegans, was shown to be thicker in the resistant "dauer" larvae than in the propagative forms (Cassada & Russell, 1975; Kondo & Ishibashi, 1978).

Correlated with this change in the ultrastructure of the epicuticle of dauer larvae, is an increased resistance to fixatives. Some dauer larvae of B. lignicolus survived 5% glutaraldehyde at 20°C for 90 minutes, while the propagative forms were fixed within 30 minutes (Kondo & Ishibashi, 1978). Likewise, the dauer larvae of C. elegans survived treatment with 1% sodium dodecyl sulphate (SDS) which killed other third-stage larvae (Cassada & Russell, 1975). Presumably the ultrastructural differences reflect a change in the composition of the cuticle which takes place without a new cuticle being formed (Riddle & Bird, 1985).

Although many alterations in the epicuticle of nematodes can be related to changed environments, and presumably represent adaptations which enhance survival under adverse conditions, not all ultrastructural variations in the epicuticle can be related to environmental influence.

For example, the outermost zone of larvae of Mermis nigrescens increased in thickness from 37 to 50 nm and appeared more distinct as the larvae aged from day 7 to day 27 (Webster & Gordon, 1974). The epicuticle of C. elegans also became progressively more distinct with age (Zuckerman, Himmelhoch & Kisiel, 1973).

Thus no single structure can be considered characteristic of the epicuticle of all nematodes. Variations in the structure of the epicuticle are apparent between species and between stages of one species, and these may be related, in part at least, to the environment in which the worm lives.

Close examination of the ultrastrutural studies relating to the cuticle of nematodes has highlighted the morphological variability of the epicuticle, but it has taken other methods to reveal changes and differences at a molecular level. The epicuticle is an antigenically active structure capable of turnover between moults, and methods generally applied to the cell surface have proved useful in identifying some molecules present on its surface.

1.2.2 Turnover of the nematode surface

A growing body of evidence suggests that nematodes control the turnover of some molecules on their surface (Maizels, Philipp & Ogilvie, 1982; Philipp & Rumjaneck, 1984). Thus molecules can be shed or replaced without a new cuticle being formed.

Release of surface molecules varies in response to environmental factors. When live infective larvae of T. spiralis, which had been labelled with radioactive iodine, were incubated in vitro, between 10 and 25% of the radiolabelled material was released into the medium over 24 hours (Mackenzie, Preston & Ogilvie, 1978). Shedding of

labelled material was enhanced to between 50 and 75% in the presence of serum or neutrophils.

Shedding of surface antigens by infective larvae may also be influenced by their metabolism. Antigenic molecules were shed from the surface of infective larvae of Ancylostoma caninum (Vetter & Klaver-Wesseling, 1978), Strongyloides ratti (Murrell & Graham, 1983) and Toxocara canis (Maizels, de Savigny & Ogilvie, 1984a; Smith, Girdwood, Kusel, Bruce & Quinn, 1981), unless the metabolism of these worms was inhibited using low temperature or sodium azide. Shedding of anti-antibody complexes by larvae of S. ratti was also inhibited by colchicine (Murrell & Graham, 1983).

Release of surface molecules is not restricted to infective-stages of parasitic nematodes, although little is known about other stages. Evidence indicates that radiolabelled material which was released into a culture medium by adult N. brasiliensis and three stages of T. spiralis originated at the surface of the worms (Maizels, Meghji & Ogilvie, 1983; Ortega-Pierres, Chayen, Clark & Parkhouse, 1983), but to my knowledge no study has directly measured the release of molecules from the surface of free-living nematodes.

The dynamic surface properties of some parasitic nemtodes appears to vary between stages of one species. Marshall and Howells (1986) reported a turnover in the surface components of third-stage larvae of Brugia pahangi. On the other hand, the surface of the fourth-stage larvae and adults of this species did not seem to alter.

Molecules might also be lost from the sheathed surface of microfilariae of B. pahangi during maturation in vivo but evidence for this indirect (Kaushal, Simpson, Hussain & Ottesen, 1984). The lectin concanavalin (Con A) bound to the surface of microfilariae derived in vitro but not to that of microfilariae taken from a host, suggesting

that the surface changed during development in the host, and molecules were probably shed. Alternatively, of course, the surface of the microfilariae could have been masked in some way.

There is little doubt that molecules can also be inserted into the surface of nematodes between moults. Surface antigens, not present on newborn larvae of T. spiralis, have been identified on older first-stage worms (Jungery, Clark & Parkhouse, 1984; Mackenzie et al., 1978). Similarly, two proteins which did not label on the surface of infective-stages of N. brasiliensis, were detected on the larvae within 18 hours of infection (Maizels et al., 1983), while third-stage larvae of Litomosoides carinii were found to acquire a new antigen after infection of a definitive host (Philipp & Worms, 1984). Furthermore, recent work suggests that when mannosyl and sialyl residues are removed from the surface of Caenorhabditis and P. redivivus using enzymes, the sugars appear to be replaced within 20 hours of being lost (Jansson, Jeyaprakash, Damon & Zuckerman, 1984).

The mechanism by which new molecules are transported to the surface of nematodes between moults is also unknown but Samoiloff (1973) presented evidence that labelled proteins can move through the cuticle of the free-living nematode P. silusiae.

Clearly the surface of nematodes undergoes dynamic changes at a molecular level without a new cuticle being synthesized. The mechanisms involved in regulating the release and expression of these molecules is still poorly understood.

1.2.3 Permeability of the epicuticle

The permeability of the epicuticle of nematodes is of interest to this discussion because it is indicative of the dynamic nature of the

epicuticle (see Section 1.2.2). This aspect of the cuticle, however, will only be dealt with briefly.

Although the cuticle of nematodes is impermeable to wide range of substances (Bird, 1971; Pappas & Read, 1975), it is permeable to water, some ions and nonelectrolytes (Marks, Thomasos & Castro, 1968; Rogers, 1961; Wright & Newall, 1976). In addition, the epicuticle of some nematodes also appears to allow passage to a variety of large molecules. Larval M. nigrescens are thought to selectively take up glucose and amino acids across the cuticle (Rutherford, 1974; Rutherford & Webster, 1974; Rutherford, Webster & Barlow, 1977). Similarly, larvae of B. pahangi have been shown to absorb glucose, amino acids and some dyes via the cuticle (Chen & Howells, 1979a; 1979b; Chen & Howells, 1981; Howells & Chen, 1981), and parasitic juveniles of Romanomermis culicivorax appear to be able to take up ferritin particles and lipid (Gordon, Burford & Young, 1982; Poinar & Hess, 1977).

Although the passage of molecules across the nematode cuticle appears to be regulated in most cases, the site at which absorption is controlled is unknown. No membrane transport proteins were detected in association with the epicuticle of B. pahangi suggesting that the passage of molecules is regulated elsewhere (Sayers, Mackenzie & Denham, 1984). The epidermis beneath the cuticle is a likely alternative candidate because it contains acid phosphatase (Howells & Chen, 1981; Maki & Yanagisana, 1979; Sayers et al., 1984).

That membrane transport proteins were not located in association with the epicuticle, is interesting because intramembrane particles have been detected within the epicuticle of some nematodes using freeze-fracture replication. Bird (1984) found particles within the epicuticle of second-stage larvae of M. javanica, and small particles were also identified within the epicuticle of N. brasiliensis when it was

freeze-fractured (Lee & Bonner, 1982). It seems possible that these particles may represent proteins involved in membrane transport.

Poinar & Hess (1977) located "pores" in the surface of R. culcivorax which they thought allowed entry to ferritin particles. The pores measured between 7 and 11 nm in diameter and appeared to fill the surface.

Thus some nematodes appear to be able to regulate the uptake of molecules through the cuticle, but it is not known whether the mechanism operates within the epicuticle or whether it is controlled within the epidermal region.

Despite the uncertainty about the involvement of the epicuticle in absorption, it is abundantly clear that the epicuticle of nematodes is a dynamic structure capable of turnover (Maizels et al., 1982; Philipp & Rumjaneck, 1984).

1.2.4 Composition of the nematode epicuticle

At this stage it is not possible to define the composition of the epicuticle, even though some proteins and carbohydrates have been identified on its surface. Results from the few studies completed to date have been inconclusive, although some insight into what the epicuticle is not composed of, has emerged. Interpretation of results is further complicated by not knowing whether different results represent true differences between species and/or stages of one species, or whether they are caused by unreliable methods.

(When discussing the work relating to the composition of the cuticle, it is important to realize that in some studies the "outer cortical zone" was referred to, without specifying whether this term relates to the epicuticle alone, or includes part of the outer cortical zone).

The outer region of the nematode cuticle is not composed of a collagen-like protein (Fujimoto & Kanaya, 1973; Leushner, Semple & Pasternak, 1979; Cox et al., 1981b), unlike the inner region which is made up of a group of such proteins (Anya, 1966; Bird, 1956; 1957; Cox et al., 1981a; 1981b; Evans, Sullivan & Piez, 1976; Fuchs & Harrington, 1970; Fujimoto & Kanaya, 1973; Josse & Harrington, 1964; Leushner et al., 1979; McBride & Harrington, 1976a; 1976b; Noble, Leushner & Pasternak, 1978; Ouazana & Herbage, 1981; Watson & Silvester, 1959; Winkfein, Pasternak, Mundry & Martin, 1985). It is insensitive to bacterial collagenase and has a different X-ray diffraction pattern and amino acid composition from collagen (Cox et al., 1981a; Fauré-Frémièt & Garrault, 1944; Fujimoto & Kanaya, 1973).

Fujimoto & Kanaya (1973) proposed that the outer 5% of the cortical zone of the cuticle of A. lumbricoides was composed of an unique protein, "cuticlin", which was distinct from collagen, elastin, resilin, keratin and fibroin. However earlier workers suggested that keratin may be included in the epicuticle (Chitwood, 1936). Moreover Murrell, Graham and McGreevy (1983) found although elastase had no effect on the binding of DEAE-Sephadex (A-50) ion-exchange beads to the surface of infective larvae of S. ratti initially, after 18 hours many of the worms were dead. Cox et al. (1981b) also noted that the outer region of C. elegans was digested by elastase.

The molecular aggregates making up the epicuticle of T. spiralis are thought to be held together by strong non-covalent hydrophobic interactions stabilized by intrachain disulphide bonds within the molecules (Clark, Philipp & Parkhouse, 1982). But published micrographs of C. elegans illustrate that the epicuticle may survive reduction, suggesting that sulphide bonds are not all important in maintaining the

structure of the epicuticle in each stage of this species (Cox et al., 1981a).

As already noted, in some instances the epicuticle resembles a plasma membrane when viewed in transverse section. Some studies involving freeze-fracture replication indicate that this ultrastructural similarity between the epicuticle and plasma membranes, extends to their composition. The epicuticle of second-stage larvae of M. javanica (Bird, 1984), of adult N. brasiliensis (Lee & Bonner, 1982) and of microfilariae of Onchocerca volvulus (Martinez- Palomo, 1978) split when freeze-fractured, indicating that it might be composed of a bilayer of lipid. But unlike a plasma membrane, the outer two layers of the cuticle of Trichinella did not split when freeze-fractured (Lee, Wright & Shivers, 1984).

The epicuticle differs from plasma membranes in the way that it responds to detergents, such as sodium dodecyl sulphate (SDS), and strong reducing agents. Plasma membranes are solubilized by such agents, but the outer portion of the cuticle of nematodes appears to withstand this treatment (Betschart, Rudin & Weiss, 1985; Cox et al., 1981b; Ouazana & Herbage, 1981; Leushner et al., 1979; Zahler & Niggli, 1977).

An example of the different properties exhibited by the epicuticle and plasma membranes comes from a study by Gutman and Mitchell (1977) which demonstrated that the surface of larvae of Ascaris suum differed from that of plasma membranes within the animal. A myeloma protein (S107), known to react with the phosphorylcholine of plasma membranes, bound to the internal membranes and lining of the intestinal tract of these larvae but did not bind to the surface of the cuticle.

Thus, apart from noting that the epicuticle is not composed of a collagen-like protein, little can be concluded about the molecules which

make it up and and the way in which they interact. The epicuticle seems unlikely to be a phospholipid bilayer, even though it may cleave in a similar way to a plasma membrane. Some proteins and carbohydrates have been identified on the surface of nematodes, however, and these results will now be discussed.

Proteins

Antigenic proteins have been located on the surface of parasitic nematodes using a technique developed for cells (Marchalonis, Cone & Santer, 1971; Parkhouse, Philipp & Ogilvie, 1981; Philipp, Parkhouse & Ogilvie, 1980a; 1980b). Living nematodes, labelled with radioactive iodine, are solubilized in buffered detergent and then the molecular weight of the labelled proteins obtained using SDS polyacrylamide gel electrophoresis. To my knowledge, apart from infective larvae of N. brasiliensis, no free-living nematodes have been examined using this method.

With few exceptions, all of the proteins that have been located on the surface of parasitic nematodes are of nematode origin. Where the dominant labelled protein was not of parasite origin, such as in microfilariae of L. carinii, Wuchereria bancrofti and Onchocerca gibsoni, it has been identified as host serum albumin (Forsyth, Copeman, Abbot, Anders & Mitchell, 1981a; Maizels, Philipp, Dasgupta & Partoni, 1984b; Philipp, Worms, McLaren, Ogilvie, Parkhouse & Taylor, 1984). It is of interest to note that host glycolipid was reported on the surface of nematodes some time ago (Ridley & Hedge, 1977; Soulsby & Coombs, 1959).

Unlike the surface of mammalian cells, few proteins dominate the surface of the species of nematode that have been studied (Maizels et al., 1982). In general, fewer than ten different molecules label on the surface of a given worm (Bashong, 1985; Forsyth et al., 1981a;

Maizels et al., 1983; Maizels et al., 1984a; 1984b; Parkhouse et al., 1981; Philipp et al., 1984). Some of the proteins adhere to lectins and so are probably glycoproteins (Clark et al., 1982; see Philipp & Rumjaneck, 1984).

Despite the limited number of surface proteins which label using the method of Philipp et al. (1980a), the proteins vary between different stages to a lesser or greater extent. In the parasites N. brasiliensis and T. spiralis, for example, all molecules that label are stage-specific (Mackenzie et al., 1978; Maizels et al., 1983; Philipp et al., 1980a; 1980b). Differences in surface molecules have also been detected between males and females of N. brasiliensis and D. viteae (Bashong, 1985; Maizels et al., 1983). In contrast, adults and larvae of the filariad worms share most surface molecules, although a few may be unique to a particular stage (Bashong, 1985; Maizels et al., 1984a; 1984b; Philipp et al., 1984).

Techniques involving radiolabelling and characterization of surface antigens of nematodes have yielded a great deal of interesting information, but several limitations should be kept in mind. Not all surface antigens are detected by the method and the specificity of alternative methods of iodination varies between different stages of a given species (Bashong and Rudin, 1982; Maizels et al., 1983; Marshall & Howells, 1985; Sutanto, Maizels & Denham, 1985). Some antigens on the surface of infective larvae of N. brasiliensis, for example, escape labelling (Maizels et al., 1983). Indeed the technique is thought to label only polypeptides containing residues of lysine and tyrosine so that polysaccharides and glycolipids probably do not label (Maizels et al., 1982). Surface components of the cuticle also differ in their solubilty under different detergent conditions (Maizels et al., 1983; Parkhouse et al., 1981; Sutanto et al., 1985).

Furthermore, although antibodies specific for solubilized labelled proteins have been shown to adhere specifically to intact worms at 4°C, it has proved impossible to check the specificity of iodination within the epicuticle because autoradiography does not offer sufficient resolution (Forsyth, Copeman, Anders & Mitchell, 1981b; Philipp et al., 1984). In some methods the label penetrates the cuticle and reaches the underlying tissue (Marshall & Howells, 1986).

Despite the technical limitations, however, it is clear that a few antigenic proteins have been demonstrated on the surface of nematodes using several methods of iodination (Maizels et al., 1983; Maizels et al., 1984; Parkhouse et al., 1981). These proteins vary between the different stages of parasitic nematodes, to a lesser or greater extent, and so reflect the ultrastructural variations of the epicuticle (see Section 1.2.1).

Carbohydrates

Carbohydrates, some of which appear to be associated with protein molecules, have been detected on the surface of free-living and parasitic nematodes using lectins (McClure & Zuckerman, 1982; Sneller, Vishnupriya & Tweedell, 1981; Zuckerman & Kahane, 1983; Zuckerman, Kahane & Himmelhoch, 1979). Lectins are proteins or glycoproteins which bind specifically to sugar residues and have been used extensively in studying the architecture of the cell surface (Sharon & Lis, 1975).

Mannose and/or glucose residues were detected on the surface of C. elegans and Caenorhabditis briggsae using the lectin concanavalin A (Con A) (Nicholson, 1974; Zuckerman et al., 1979). With Con A conjugated to hemocyanin molecules, McClure & Zuckerman (1982) established that the distribution of glucose/mannose on C. elegans and M. incognita was uneven, with most binding sites occurring in the head region. Con A has also been found to bind to the surface of

microfilariae of B. pahangi and Dirofilria immitis, but not to the adults or embryonated eggs of either species (Sneller et al., 1981).

Saccharides containing N-acetyl glucosamine/N-acetyl neuraminic acid/N-acetyl galactosamine were located on several species of nematode (Sneller et al., 1981; Zuckerman et al., 1979) using wheat germ agglutinin (WGA). Indeed WGA was the only lectin out of several tested that bound to the adults, microfilariae and embryonated eggs of both B. pahangi and D. immitis and to infective larvae of Brugia malayi (Kaushal et al., 1984; Sneller et al., 1981). It also labelled eggs, first-stage larvae, young and old adults of C. elegans (Zuckerman & Kahane, 1983). When the sheathed surface of B. pahangi was labelled with Con A and WGA, the label was progressively lost during maturation (Furman & Ash, 1983).

N-acetyl-galatose has been identified on the surface of three plant parasitic nematodes using soybean (SBA) agglutinin (Nicholson, 1974; Spiegel, Cohn and Spiegel, 1979). Unfortunately no information about the distribution of binding on any of the worms was given.

The lectin Ricinus communus agglutinin (RCA) was used to locate β-D-galactose/ N-acetyl-galactosamine on the surface of C. elegans and C. briggsae (Zuckerman et al., 1979). These sugars were also found on the surface of larval and adult Tylenchulus semipenetrans and adult Xiphinema index (Spiegel, Cohn & Spiegel, 1982; Spiegel, Robertson, Himmelhoch & Zuckerman, 1983).

Sialyl residues were detected along the entire length of the outer body wall of Helicotylenchus multicinctus, M. javanica and T. semipenetrans but were restricted to the tail and head regions in X. index. No sialyl residues were detected on larvae of H. multicinctus (Spiegel et al., 1982).

Some of these carbohydrates appear to be associated with protein. Trypsin and pronase blocked labelling of T. semipenetrans (Spiegel et al., 1982) but pronase had no effect on the binding of Con A, WGA and RCA to the surface of either C. elegans or C. briggsae (Zuckerman et al., 1979).

Surface charge

A feature shared by the surface of nematodes and that of cells is the presence of a net negative charge. In cells this negativity is often associated with the presence of sialic acid which is located at the terminal position of the carbohydrate chains making up the glycocalyx (Lloyd, 1975).

There is some conflicting evidence about the nature of the molecular groupings responsible for the negative charge at the surface of nematodes. Himmelhoch and Zuckerman (1978) found that neuraminidase had no effect on the binding of cationized ferritin to the surface of C. elegans. Nor had neuraminidase any effect on the binding of colloidal iron to the surface of C. elegans (Himmelhoch, Orion & Zuckerman, 1979). Similarly sialyl residues on H. multicinctus and M. javanica were not susceptible to treatment with neuraminidase but those on T. semipenetrans were (Spiegel et al., 1982). Work by Hudson and Kitts (1971) intimated that sialic acid is present on the surface of the lungworm, Protostrongylus stilesi. These workers found that cells adhered to the surface of the larvae, unless the larvae had been pretreated with neuraminidase.

In general, an even distribution of cationized ferritin has been found to bind to the surface of nematodes (Himmelhoch et al., 1979; Murrell et al., 1983) but in M. javanica, cationized ferritin did not bind at the base of the annules (Himmelhoch et al., 1979). It is interesting to note that while cationized ferritin bound to the surface

of sheathed B. pahangi, none was observed on the surface of microfilariae of D. immitis (Hammerberg, Rikihisa & King, 1984).

A number of other substances have also been used to demonstrate a net negative charge on the surface of nematodes. Thorium dioxide (Bonner, Menefee & Etges, 1970), colloidal iron (Himmelhoch et al., 1979) and alcian blue stain the surface of nematodes (Cherian, Stromberg, Weiner & Soulsby, 1980; Lewis & Knight, 1977). In addition, net negativity has been demonstrated on the surface of C. briggsae, M. javanica, D. immitis, B. pahangi and S. ratti using the ruthenium red ion (Cherian et al., 1980; Hammerberg et al., 1984; Himmelhoch et al., 1979; Murrell et al., 1983). Although ruthenium red is some times claimed to be a stain specific for acidic mucopolysaccharides, there is no evidence to support this assumption (Lewis & Knight, 1977).

Function of surface molecules

Although a few different molecules have been identified on the surface of nematodes, very little is known about their function. However, some insight into the possible role of such molecules is provided by an recent interesting experiment. Treatment of C. elegans and P. revidivus with sialidase or mannosidase completely inhibited chemotactic responses of these nematodes to a source of attractants (Jansson et al., 1984). Thus mannosyl and sialyl residues on the cuticle are thought to be important in the detection of chemotactic signals, and indirect evidence suggests that these sugars may occur near the sensilla (Zuckerman, 1983).

As can be seen from the foregoing discussion, very little is known about the composition of the nematode epicuticle. Although the epicuticle may cleave when freeze-fractured it clearly has different properties to other phospholipid membranes. Not only is the epicuticle

often multilaminate, but it also contains fewer surface proteins which label with radioactive iodine, and is more resistant to a variety of chemicals.

As a preliminary to the investigation into the nature of the epicuticle of N. dubius, the ultrastructure of the whole cuticle of this nematode was examined.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 THE EXPERIMENTAL ANIMAL : N.dubius

2.1.1 Classification

The nematode under study belongs to the order Strongylida and is closely related to a number of nematodes of medical and economic importance. Although classified as Heligmosomum polygyrum (Dujardin, 1845) Raillet and Henry, 1909, this name is rarely used (Chabaud, 1965; Durette-Desset, 1968; Raillet & Henry, 1909; Skrjabin, 1952). Of the synonyms that have also been suggested, Nematospiroides dubius (Baylis, 1926) is one of the more common and will be used throughout this study.

The strain of N. dubius was obtained originally from the McMaster Laboratory, CSIRO, Sydney, but has been maintained in the Department of Zoology, University of Adelaide for the past 20 years.

2.1.2 Life History

N. dubius parasitizes the house mouse, deermouse and other wild rodents of North America and Europe (Levine, 1968). Feral Mus musculus, for example, commonly harbour light infections of between 1 and 30 worms (Forrester, 1971), while infections of between 150 and 300 worms can be maintained in the laboratory (Baker, 1954; 1955). Different strains of mice differ in the degree to which they are susceptible to infection by N. dubius (Baker, 1954; Cypess & Zidian, 1975; Liu, 1965a; Spurlock, 1943).

The life cycle of N. dubius has been closely studied (Baker, 1954; Bryant, 1973; Ehrenford, 1954; Fahmy, 1956; Spurlock, 1943; Sukhdeo, O'Grady & Hsu, 1984). The sexually mature adult worms, which are spirally coiled, live entwined between and around the villi of the small intestine of mice with their anterior ends buried in the crypts of Lieberkühn (Baker, 1954). Longitudinal ridges are thought to help the worms attach to the villi (Kleinschuster, Hepler & Voth, 1978). The sexes are separate. The female, which has a truncate tail, measures about 13 mm in length and can be easily distinguished from the shorter male worm which has a distinctive bursa with asymmetrical lateral lobes in the tail region (Levine, 1968).

Eggs produced by the adult females appear in the faeces of the host and hatch within 37 hours to liberate first-stage larvae (Bryant, 1973). A second moult takes place after a further 28 or 29 hours and is followed by a partial moult, some 17 to 20 hours later. Thus, third-stage larvae, which are infective, remain ensheathed in the second-stage cuticle. The infective larvae are active but do not feed. When ingested by a suitable host the third moult (exsheathment) is completed (Bryant, 1973).

Following ingestion about one-third of the larvae penetrate the gastric mucosa of the fundic region where they remain for between 24 and 36 hours (Liu, 1965a; Sukhdeo et al., 1984). The other larvae, however, move quickly through the stomach and into the intestine where they are always found in the mucosa and submucosa within 24 hours of infection. Between the first and third days of infection, the larvae move to, and penetrate the muscularis externa (Sukhdeo et al., 1984). In the natural host, worms lie coiled, bounded not by connective tissue, but by cells of the circular muscle layer of the muscularis externa (Cross, 1960; Sukhdeo et al., 1984). Although sexual differentiation is apparent by

70 hours after infection, the fourth moult does not take place until 26 hours later (Bryant, 1973).

The final moult occurs 6 to 7 days after infection and by day 8 the worms begin to re-emerge into the lumen of the intestine, producing eggs within 2 days (Bryant, 1973). The adults remain in the intestine for, on average, 8 months but infections of between 4 and 11 months have been recorded (Cross, 1960).

Single light infections of N. dubius do not appear to be recognized by the host but repeated experimental infections will induce immunity (Bartlett & Ball, 1972; Chaicumpa, Prowse, Ey & Jenkins, 1977; Dobson, 1982; Panter, 1969). Light infections are usually well tolerated by the host, but peritonitis (Spurlock, 1943; Baker, 1954), anaemia (Baker, 1954; 1955) and damage to intestinal epithelium have been observed in heavy infections (Baker, 1954; Liu, 1965b; Spurlock, 1943).

The life history of N. dubius, therefore, makes it amenable to experimental manipulation. The period of prepatency is short, while patency is prolonged. Because no intermediate host is required, the nematode can be readily maintained in the laboratory (Jones & Weinstein, 1957; Sommerville & Weinstein, 1964) where it has been used in studies on cuticle formation and exsheathment (Bonner & Weinstein, 1972a; 1972b; Sommerville & Bailey, 1973). In addition, Bartlett and Ball (1972) suggested that a population of mice repeatedly infected with N. dubius be used as a model for studying the effect of endemic hookworm on human populations.

2.2 MAINTENANCE IN THE LABORATORY

2.2.1 Routine infections

Albino female mice, LACA strain, weighing between 20 and 25 grams, were infected with 250 larvae, introduced into the oesophagus in 0.05 ml of water using a blunt needle.

Where third-stage larvae were required for routine infections, they were grown following the method of Burren (1980). Faeces from mice infected with *N. dubius* were collected overnight on moist paper and mixed into a paste using tap water. A small amount of this faecal mixture was placed on a double thickness of wet filter paper (Whatmans Ashless 542) that was supported by an upturned watchglass in a Petri dish. The cultures were kept moist at 21°C and third-stage larvae were collected from the thin film of water surrounding the filter paper from 9 to 15 days later. Larvae were washed and stored in shallow layers of distilled water at 4°C.

2.2.2 Recovering parasitic stages

To collect third-stage larvae, mice infected with between 300 and 600 infective larvae (see Section 2.2) were killed 2 to 83 hours later. The stomach and small intestine were removed, slit open, washed and incubated in 0.85% sodium chloride at 37°C for about 30 minutes, after which time the worms began to emerge from the tissue.

To collect adult worms, the small intestine of the mouse was removed and immersed in 0.85% sodium chloride. The intestine was slit open, and the adults picked from the lumen of the intestine using a hooked tungsten needle. Fourth-stage larvae were obtained either by slitting open cysts that were clearly visible on the outside of the

intestine, or by collecting those larvae that emerged unaided from the cysts into the salt solution.

2.2.3 Harvesting free-living stages

In a few experiments it was necessary to harvest large numbers of eggs of N. dubius for axenic cultivation of first- and second-stage larvae.

Eggs of N. dubius were isolated using a modification of the method involving sucrose gradient centrifugation developed by Marquardt (1961). A faecal mixture (see Section 2.2.1) was sieved and washed 3 times by centrifugation at 2400 rpm for 5 minutes. Some of the mixture was added to centrifuge tubes containing a gradient of sucrose concentrations made up of sucrose solutions in the ratios of 1:2, 1:4, 1:6 and 1:8 (sucrose : water). The tubes were centrifuged for 20 minutes at 2400 rpm, without braking and the eggs were collected from the 1:4 layer, just above the 1:2 junction. The eggs were washed twice by centrifugation and sterilized by a 15 minute exposure to a hypochlorite solution made by diluting 0.65 ml of "Milton" (1% sodium hypochlorite; Richardson-Merrell Pty. Ltd., Australia) to 20 ml with distilled water.

Following sterilization, eggs were washed twice in sterile distilled water. Sterility was tested by placing a drop of the egg suspension in thioglycollate medium (Difco Laboratories, USA) at 37°C. Sterile methods were used for subsequent culturing of eggs and first-stage larvae.

Sterile eggs were added to screw-capped Erhlenmeyer flasks, each containing 1.9 ml of Krebs-Ringer salt solution buffered with 0.1M phosphate buffer to pH 6.0 (Umbreit, Burris & Stauffer, 1972) and including 0.1 ml of Kanamycin (Bristol Laboratories, Australia; final

concentration 0.05 mg/ml). Flasks were incubated overnight at 26°C. First-stage larvae emerged from the eggs 20 to 24 hours later.

The method used to obtain a synchronously developing population of second-stage larvae followed that of Mauro and Weinstein (1979). First-stage larvae were added to a culture medium made up of 1.9 ml of E. coli in distilled water and 0.1 ml of Kanamycin (final concentration of 0.05 mg/ml) in an Ehlenmeyer flask and incubated at 26°C for 40 hours, when substantial numbers of second-stage larvae appeared. E. coli (K12 strain), obtained from the Deptartment of Microbiology, University of Adelaide, were killed by exposure to 2% formalin at room temperature for 45 minutes. After being washed 3 times in sterile saline by centrifugation, bacteria were resuspended in distilled water to give an optical density of 0.77 to 0.8 at 650 nm.

2.3 GENERAL ELECTRON MICROSCOPE PROCEDURES

Nematodes used in this study were routinely fixed in 3% glutaraldehyde buffered with 0.1M Sörensen's phosphate buffer at pH 7.2 for between 90 and 120 minutes at 4°C. During the first 30 minutes in glutaraldehyde, worms were cut into small pieces using either a glass knife or tungsten needles. Following primary fixation, worms were thoroughly washed in 3 changes of cold Sörensen's phosphate buffer at pH 7.2 over 30 minutes. Worms were post-fixed in 1% osmium tetroxide, made up in the same buffer at 4°C for 1 hour. After fixation, worms were washed briefly in the Sörensen's phosphate buffer and dehydrated with ethanol as follows: 10 minutes in each of 30%, 50%, 70%, 90% and 95%, followed by 3 changes of absolute ethanol and then 10 minutes in a 1:1 mixture of absolute ethanol and propylene oxide (1,2-epoxypropane; Ajax Chemicals, Australia). Final dehydration was in

2 changes of propylene oxide over 30 minutes.

The tissue was infiltrated overnight under vacuum in a mixture (1:1) of propylene oxide and Spurr low-viscosity embedding medium (Polysciences Inc., USA) at room temperature and transferred to fresh resin for a further 8 to 30 hours before being embedded.

Sections were cut on a Reichert OM3 ultramicrotome using glass knives and mounted on copper grids. They were stained routinely with 5% aqueous uranyl acetate at 50°C for 5 or 10 minutes and counterstained with lead citrate (Reynolds, 1963) for 2 minutes at room temperature. Material was viewed and photographed in a Jeol 100S electron microscope. In general, sections taken from at least 5 worms from each treatment were examined.

Using a line grating (2160 lines per mm) it was established that a maximum error of 4% occurred when determining the actual magnifications of the negatives, regardless of whether the negatives were taken on the same day or weeks apart. In calculating the actual dimensions of a given structure from a negative, therefore, measurements were recorded as a range to take into account a maximum error of $\pm$ 4%. Unless otherwise stated, each measurement represents the mean from at least 10 micrographs.

CHAPTER 3

ULTRASTRUCTURE OF THE CUTICLE OF N. dubius

3.1 INTRODUCTION

The life cycle of nematodes is punctuated by four moults. During moulting, the old cuticle separates from the underlying epidermal membrane and a cuticle that differs in composition and ultrastructure is synthesized beneath it (Bird, 1971; Bonner & Weinstein, 1972a; Lee, 1970; Cox et al., 1981a; Sommerville, 1982).

The structure of the nematode cuticle may be related, in part at least, to the environment in which the worm lives. In nematodes that are subject to abrupt changes in environmental conditions, such as parasitic nematodes, the cuticle often exhibits diversity of structure between stages (Davey, 1965; Lee, 1965; Lee, 1970; Thust, 1966; Watson, 1965). In contrast, the cuticle of free-living nematodes shows less variation between stages (Cox et al., 1981a; Samoiloff & Pasternak, 1968).

Few ultrastructural studies have compared the cuticle of each stage of a nematode species (Cox et al., 1981a; Kozek, 1971; Samoiloff & Pasternak, 1968). To my knowledge, no study has investigated the structure of the cuticle of a nematode which occupies both free-living and parasitic niches during its life cycle. In this study, the ultrastructure of each cuticle produced by N. dubius was examined. Particular attention was focused on the cuticle of the free-living stages and on the ultrastructure of the epicuticle.

3.2 MATERIALS AND METHODS

Worms were collected and fixed as described in Sections 2.2.3 and 2.3. First-stage larvae were fixed uncut, but second-stage worms were cut into halves using a glass knife. These stages could be easily distinguished; second-stage larvae were coated with E. coli from the culture medium but no bacterial cells were seen on first-stage larvae (see Chapter 6).

In some instances, infective larvae were artificially exsheathed prior to fixation. Larvae were added to 3 ml of 0.85% sodium chloride (adjusted to pH 3 with 3N hydrochloric acid) which had been pre-gassed with 5% carbon dioxide in air for 3 minutes and pre-heated to 37°C (Sommerville & Bailey, 1973). Following a 30 minute exposure to these conditions, the exsheathed larvae were washed in 0.1M Sörensen's phosphate buffer pH 7.2, and prepared for transmission electron microscopy (see Section 2.3).

The method used to freeze-fracture the cuticle of infective worms is described in Section 5.2.

3.3 RESULTS

The cuticle of N. dubius showed marked ultrastructural differences between stages (see Fig. 3.16 for summary).

3.3.1 First-stage cuticle

The first-stage cuticle measured between 100 and 120 nm in thickness (Fig. 3.3). Three distinct zones were detected when this

cuticle was viewed in transverse section; the epicuticle, and the cotical and basal zones.

The outermost zone or epicuticle was composed of a trilaminate structure between 14 to 17 nm thick (Fig. 3.4). The inner layer of the epicuticle was wider and more dense than the outer layer (6.6 to 8.8 nm compared with the 2.5 to 3.2 nm). These two dense layers were separated by an electron-lucent region. The surface of the epicuticle appeared smooth (Fig. 3.4).

Beneath the epicuticle, the cortical zone made up the bulk of the cuticle. This zone was between 40 and 50 nm thick, and appeared granular and homogeneous in composition.

In contrast to the cortical zone, the basal zone was composed of electron-dense parallel lines measuring 45 to 55 nm in height and about 4.5 nm in width which were separated by an electron-lucent zone approximately 15 nm wide. (Fig. 3.4). Where the cuticle folded to form the lateral alae, an additional layer of fibres was present in the basal zone beneath the striated material which was upfolded into the cuticular ridges (Fig. 3.4).

Lateral specializations of the cuticle, the lateral alae, ran along the length of the first-stage larvae. Each was composed of five ridges, with the middle three ridges being more prominent than the outer ones (Figs 3.1 and 3.4).

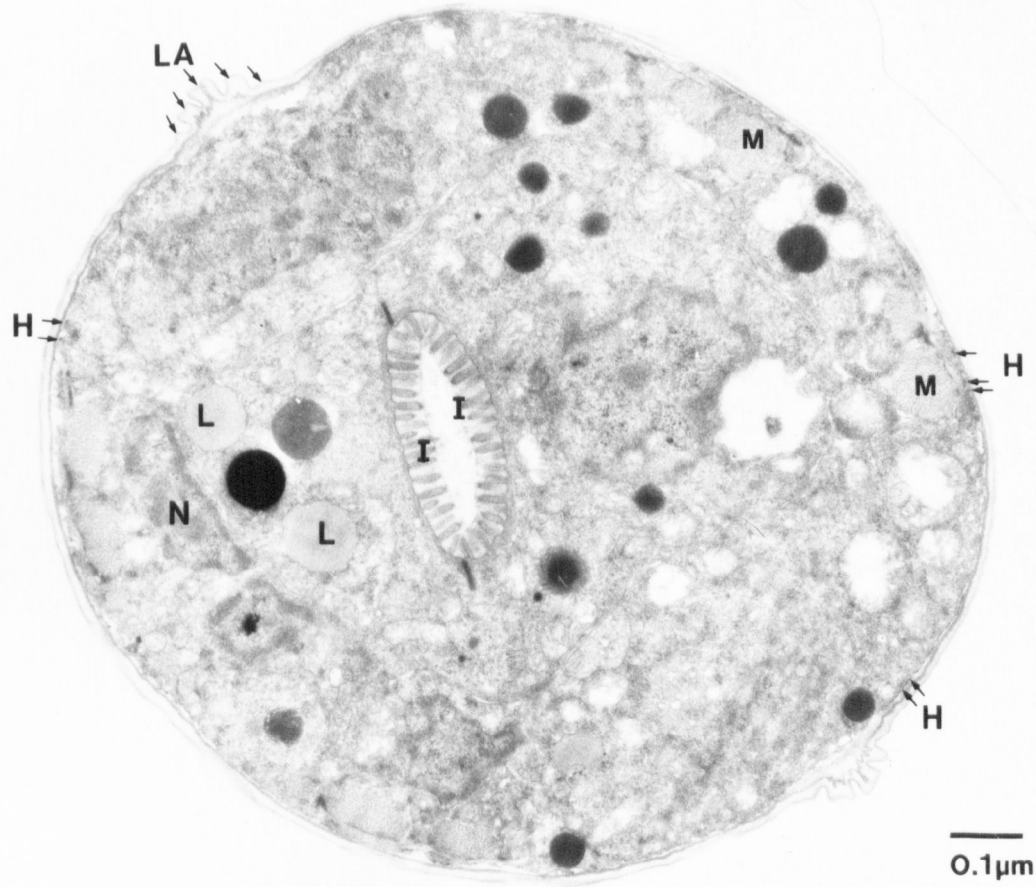
Cuticle and epidermis appeared to be attached at dense regions or hemidesmosomes, and the epidermal membrane was visible as a continuous structure beneath the cuticle.

3.3.2 Second-stage cuticle

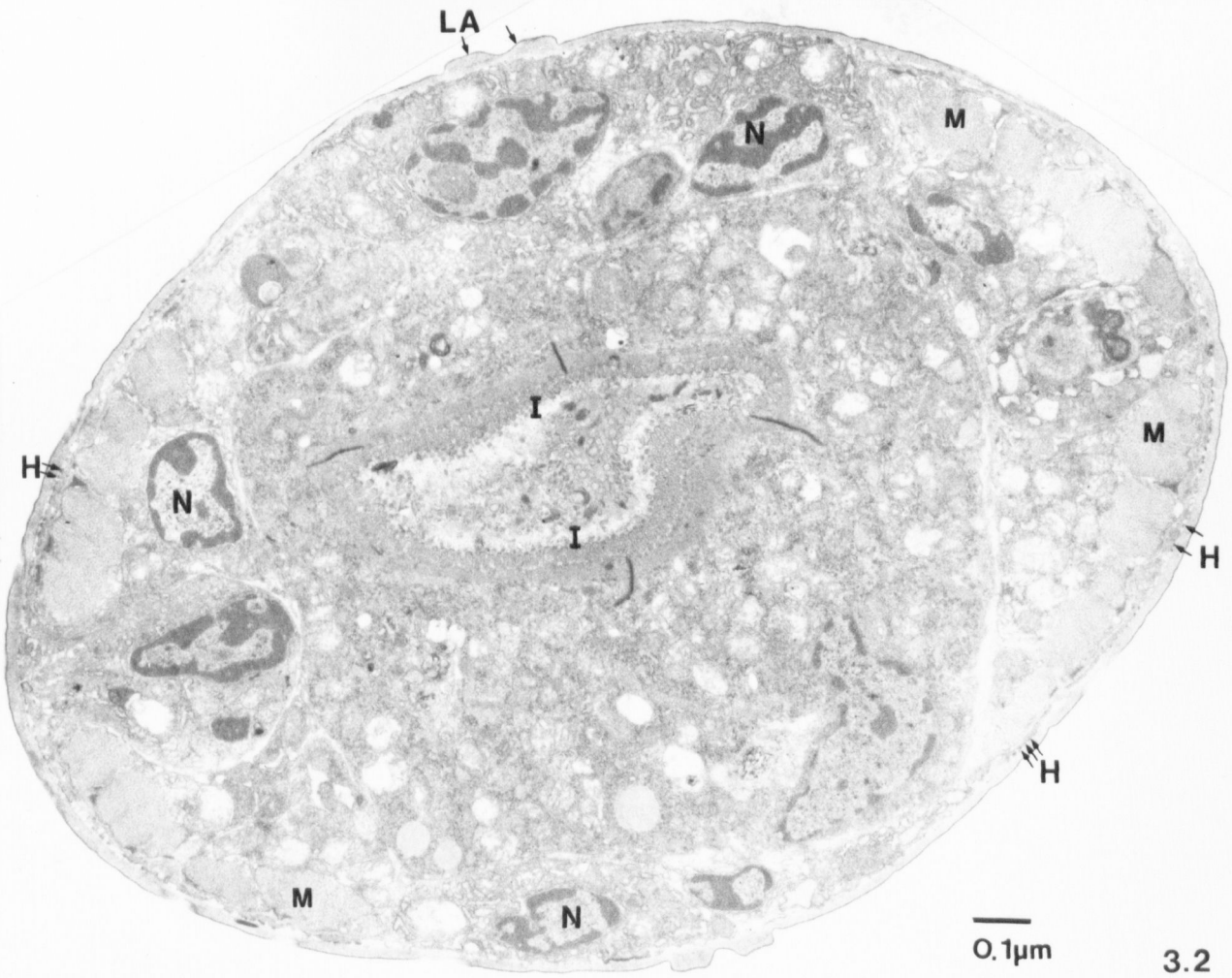
The second-stage cuticle was slightly thicker than that of first-stage larvae, measuring between 140 and 200 nm in thickness when

Fig. 3.1: Transverse section through a first-stage larva of Nematospiroides dubius. A specialization of the cuticle, the lateral ala (LA), runs along each side of the animal, and is composed of 5 ridges. Also visible are muscle fibres (M), active nuclei (N), lipid droplets (L) and intestine (I). Note the dense patches or hemidesmosomes (H) between the cuticle and epidermis in some regions.

Fig. 3.2: Transverse section through a second-stage larva of Nematospiroides dubius. A specialization of the cuticle, the lateral ala (LA), runs along each side of the animal, and is composed of 2 broad, flattened ridges. Note the muscles (M), nuclei (N) and the intestine (I). Hemidesmosomes (H) can be identified between the cuticle and the epidermis.



3.1



3.2

Figs 3.3 and 3.4: First-stage cuticle of Nematospiroides dubius.

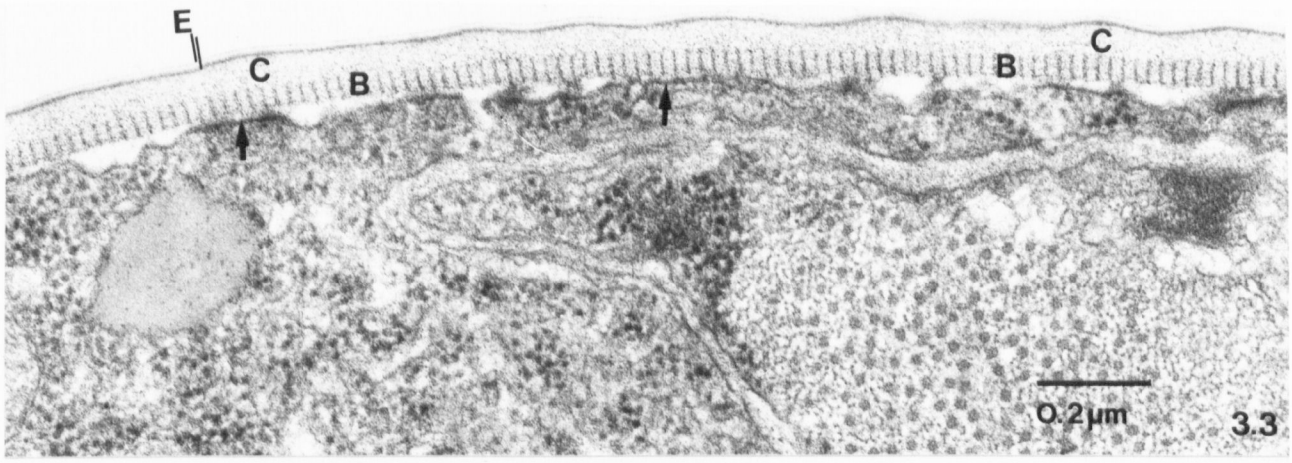
Fig. 3.3: The cortical zone (C) is granular. Striated material is present in the basal zone (B). The outer layer of the trilaminate epicuticle (E) is less dense than the inner one. Note the epidermal membrane (→).

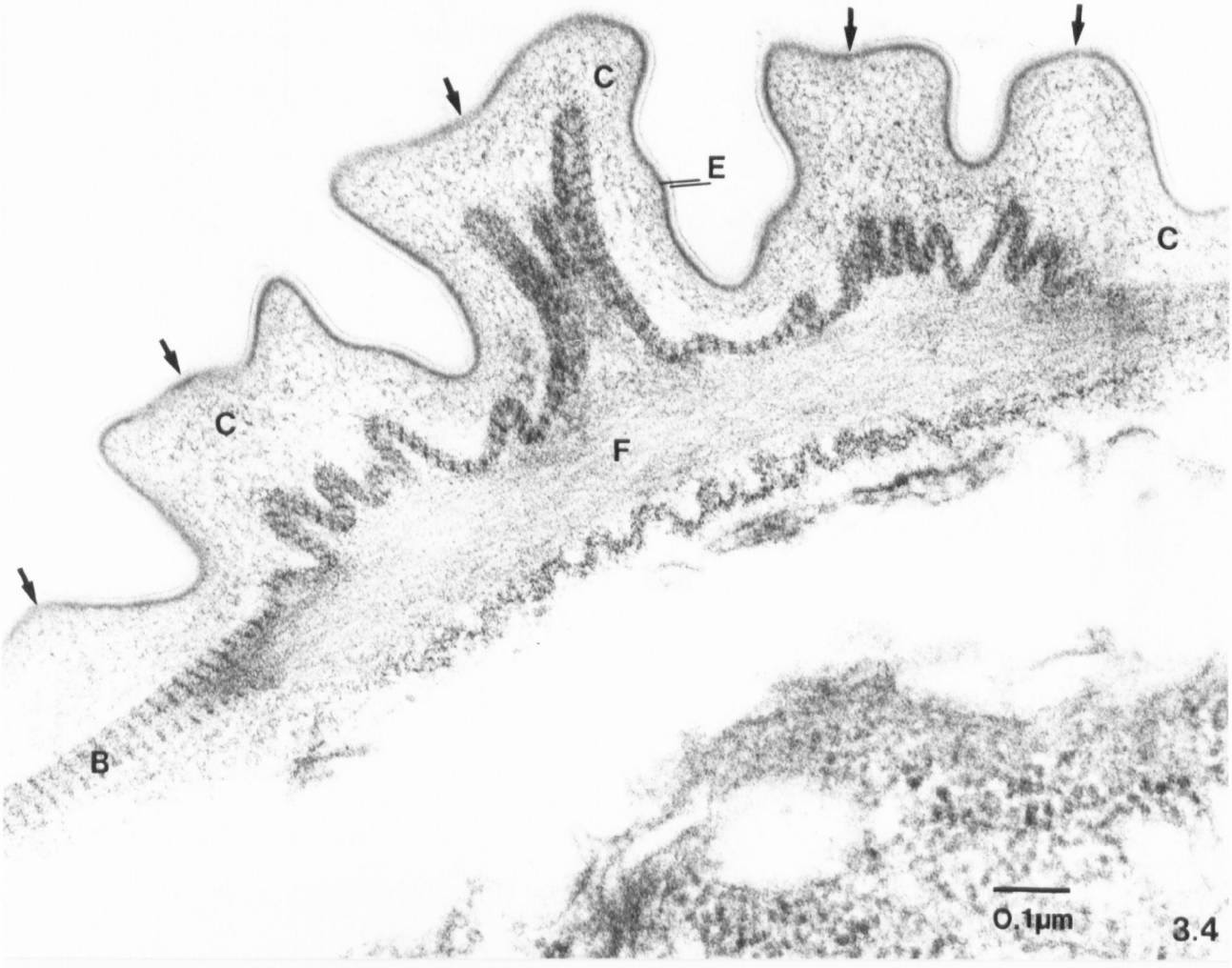
Fig. 3.4: The lateral ala is composed of 5 ridges (→). Striated material of the basal zone (B) is upfolded into the ridges and additional fibrous material (F) is present. Note the trilaminate epicuticle (E) above the granular cortical zone (C).

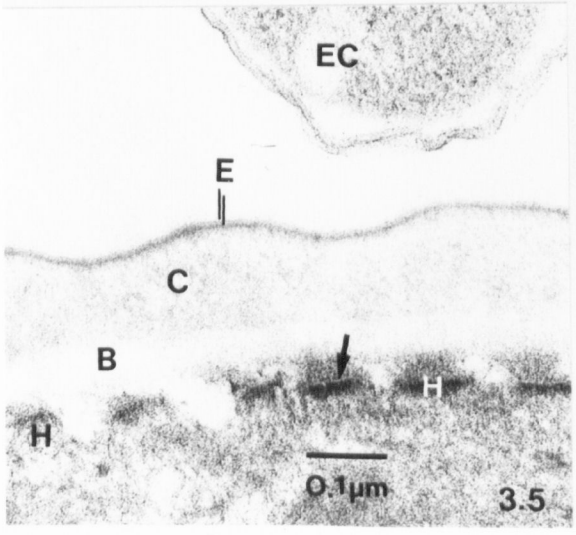
Figs 3.5 and 3.6: Second-stage cuticle of Nematospiroides dubius which is bounded by a trilaminate epicuticle (E). Granular material in the cortical zone (C) is more dense than the basal zone (B).

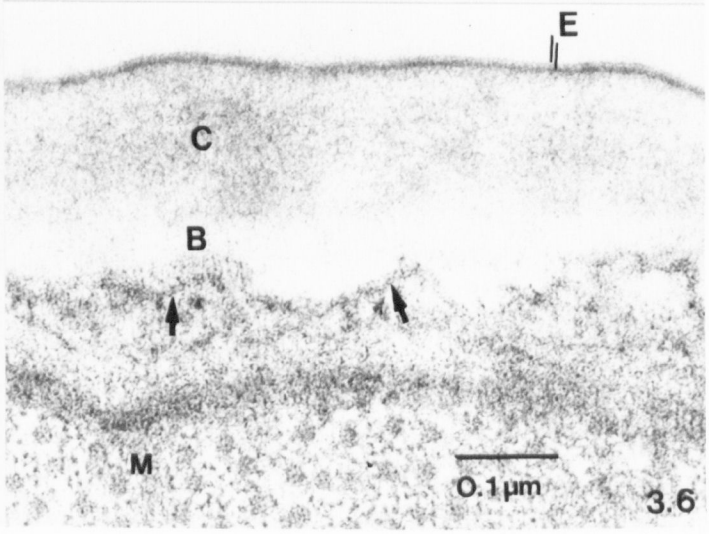
Fig. 3.5: Escherichia coli (EC) adhered to the surface of the second-stage cuticle of this larva grown in culture. Note the prominent dense hemidesmosomes (H) and the epidermal membrane can be detected as a trilaminate structure in some regions (→).

Fig. 3.6: At high magnification the E can be resolved as a trilaminate structure in which the innermost line is more dense than the outer one. The epidermal membrane can be detected in some regions (→). The muscle (M) has been cut at right angles to the fibres.


E
C
B
B
C
0.2µm
3.3


C
E
C
C
F
B
0.1µm
3.4


EC
E
C
B
H
H
0.1µm
3.5


E
C
B
M
0.1µm
3.6

viewed in transverse section. At low magnification, the second-stage cuticle exhibited little structure apart from lateral alae (Fig. 3.2). Second-stage larvae were processed for sectioning on 6 occasions to verify that the apparent lack of structure within the cuticle was not caused by inadequate techniques.

At high magnification, the epicuticle of the second-stage cuticle was less clearly delineated than that of the first-stage larvae (compare Figs 3.5 and 3.6 with Figs 3.3 and 3.4). It measured between 7 and 9 nm in thickness and was difficult to detect using a magnification of less than x100,000. No surface coat was present in sections stained with uranyl acetate and lead citrate, but E. coli from the culture medium adhered to the outer face of the cuticle (Figs 3.5 and 3.6).

The bulk of the cuticle was made up of a granular cortical zone, but a less dense region measuring between 45 and 60 nm in thickness was present in the basal region of the cuticle (Figs 3.5 and 3.6). The lateral alae were composed of 2 flattened ridges.

Hemidesmosomes were apparent between cuticle and epidermis (Figs 3.2 and 3.5). The epidermal membrane could be detected but appeared discontinuous in some regions (Figs 3.5 and 3.6).

Following the second (partial) moult, the second-stage cuticle forms the protective sheath of the infective larva. The sheath, which measured between 300 and 400 nm in thickness, was about twice as thick as the cuticle of early second-stage larvae (Fig. 3.7).

3.3.3 Third-stage (infective) cuticle

The cuticle of infective-stage larvae was separated from the environment by the overlying second-stage sheath (Fig. 3.7) and measured between 290 and 370 nm in thickness. Three distinct zones could be distinguished when sections of the third-stage cuticle were viewed in

transverse section, namely the epicuticle, the cortical zone and the basal zone.

The epicuticle was a multilaminate structure measuring between 22 and 26 nm in thickness (Figs 3.7 and 3.11). At least 5 electron-dense lines, separated by electron-lucent zones could be detected in ultra thin sections of the epicuticle (Fig. 3.11), but when freeze-fracture replicas of cross-fractured epicuticle were viewed, only 2 parallel lines were visible (Figs 3.8 and 3.9). No surface coat was detected.

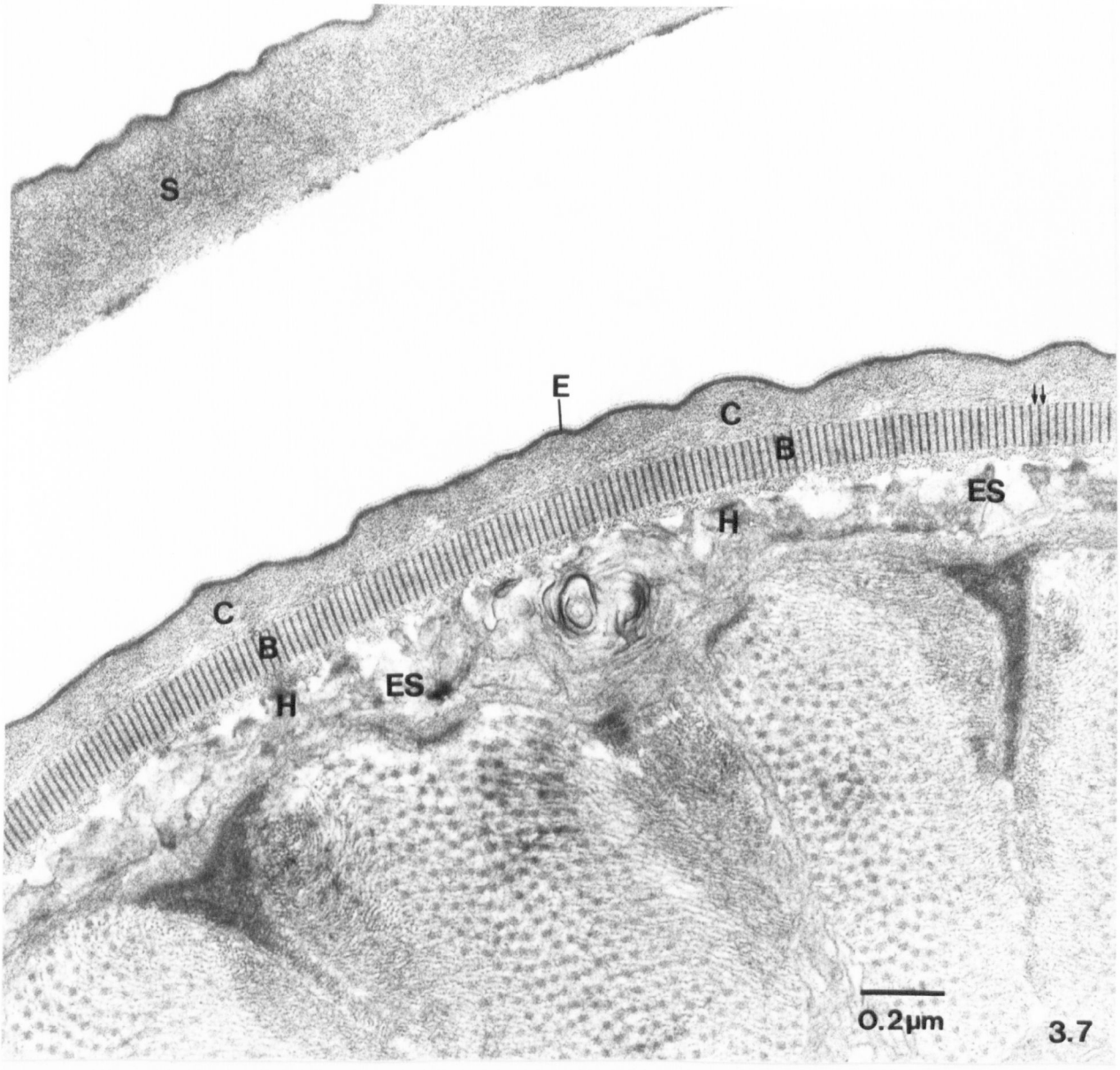
The cortical zone was between 120 and 180 nm thick and appeared granular and amorphous. In contrast, the basal zone was made up of a series of parallel electron-dense lines which measured between 100 and 115 nm in height and about 7 nm in width. The dense lines were separated by a less dense zone which was between 12 and 15 nm wide (Fig. 3.7). Within this less dense region, a fine electron-dense line, parallel to the striated material could be detected (Fig. 3.7). A lattice-like arrangement of the material forming the basal zone was demonstrated using freeze-fracture replication (Figs 3.9 and 3.10), and in oblique thin sections (see Fig. 7.18).

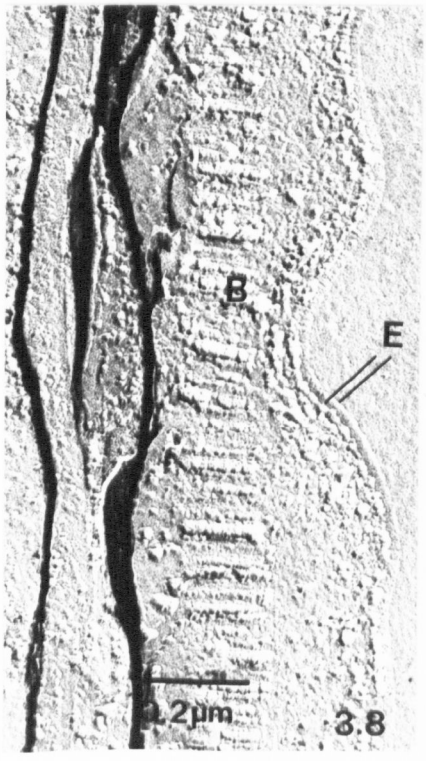
Several modifications to the structure of the third-stage cuticle occurred in the lateral regions (Fig. 3.11). The striated material did not extend across the alae but tapered into a convoluted structure on each side of the alae. A dense band of fibres could be detected between these convoluted ends indicating that the striated material might still be present but at a different orientation. Two additional layers, one fibrous and one granular, were present in the basal zone beneath the striated material. Furthermore, the core of the lateral alae was less dense than the remainder of the cuticle.

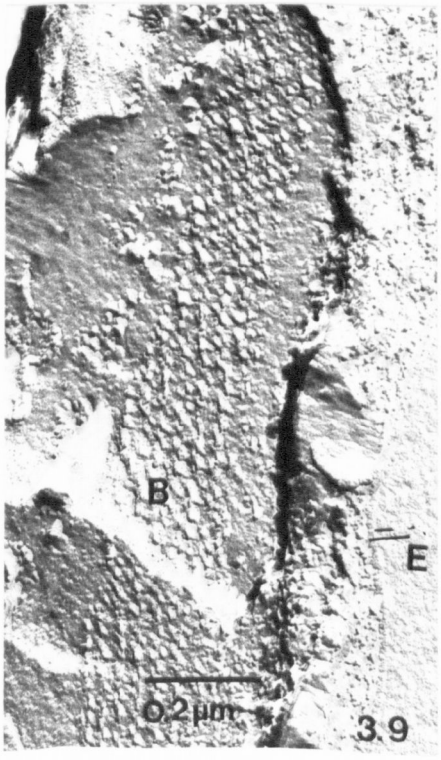
The third-stage cuticle was joined to the epidermis at

Fig. 3.7: Transverse section through an infective larva of Nematospiroides dubius, enclosed within the second-stage sheath (S). The third-stage cuticle is composed of a multilayered epicuticle (E), a granular cortical zone (C), and a basal zone (B) containing striated material. Between the dense parallel lines making up the striated material are less dense, thinner lines (→). The epidermal surface (ES) is highly convoluted and attached to the cuticle at hemidesmosomes in some regions (H).

Figs 3.8 to 3.10: Freeze-fracture replicas of the third-stage cuticle of Nematospiroides dubius. Note the appearance of the striated material of the basal zone (B) when fractured through different planes. The epicuticle (E) appears trilaminate when cross-fractured (Figs 3.8 and 3.9).


S
E
C
B
ES
H
C
B
H
ES
0.2µm
3.7


B
E
0.2µm
3.8


B
B
E
0.2µm
3.9

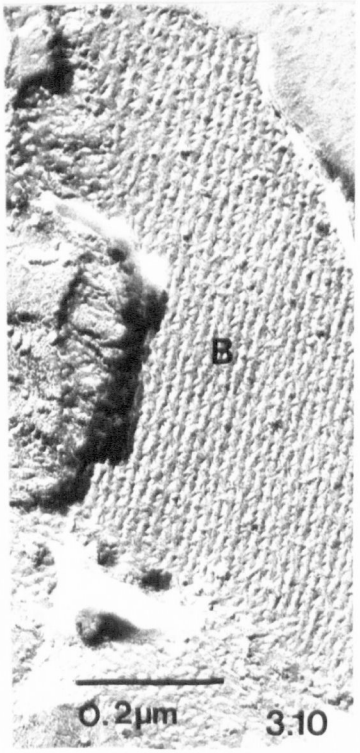
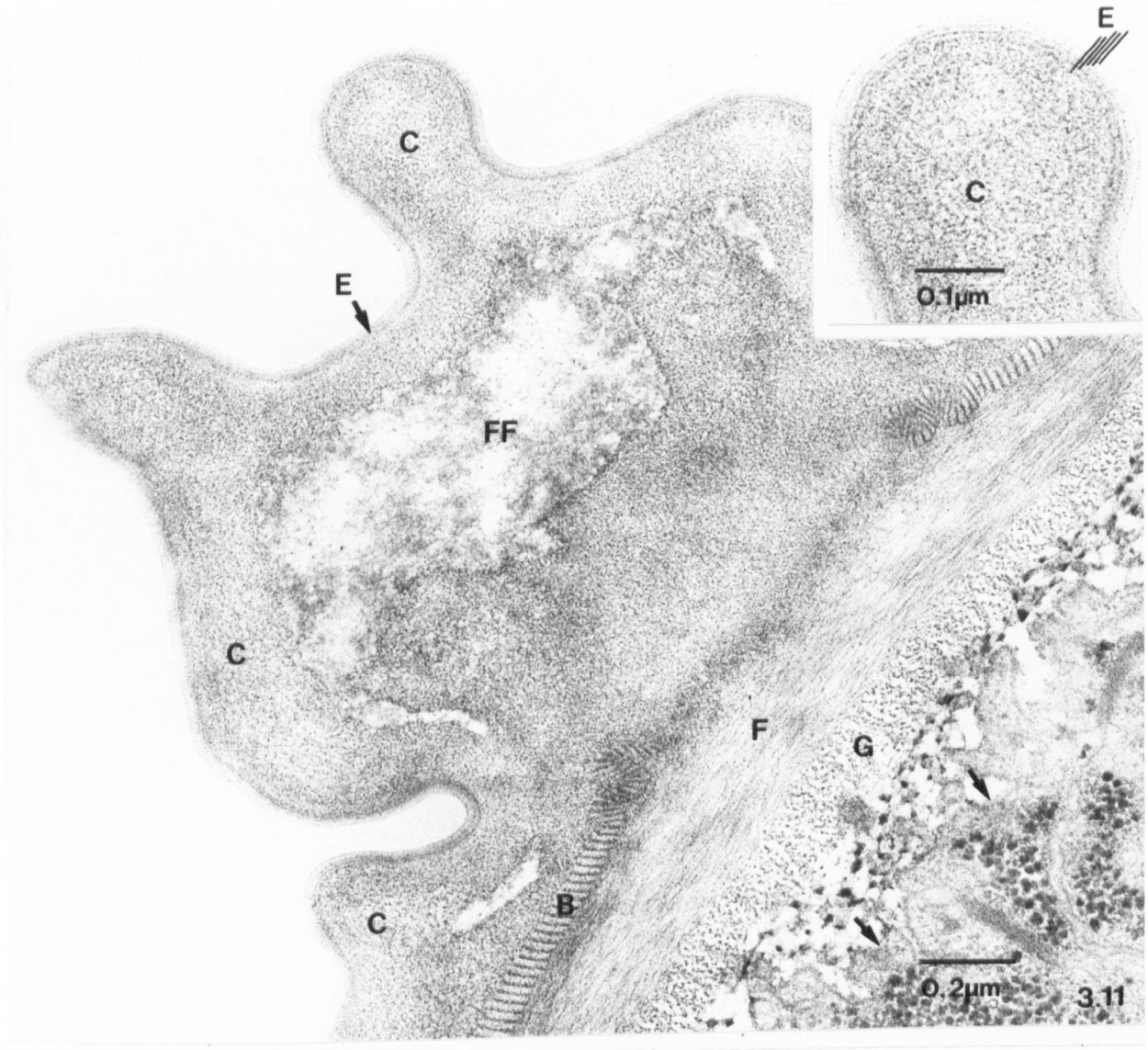
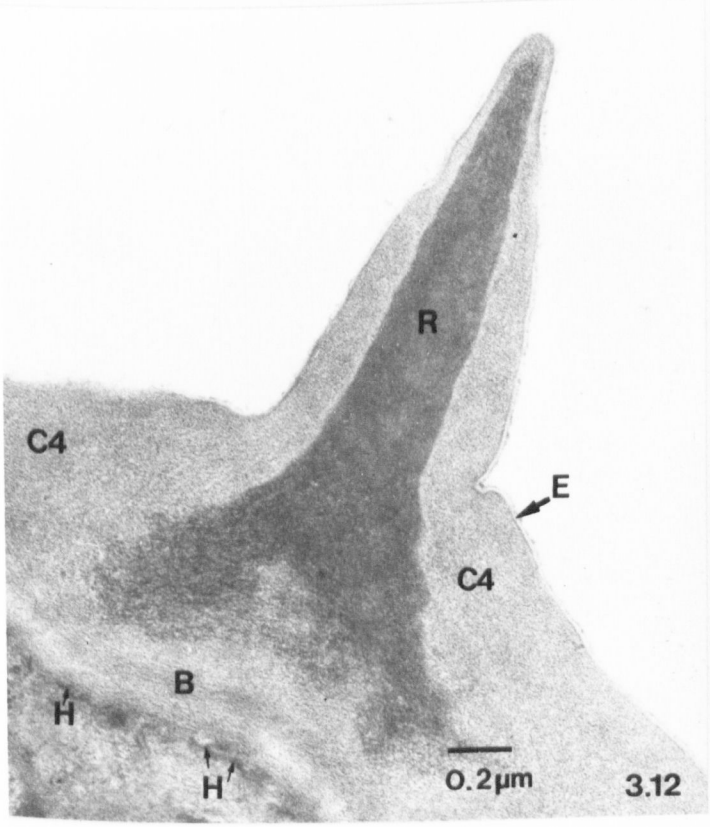

B
0.2µm
3.10

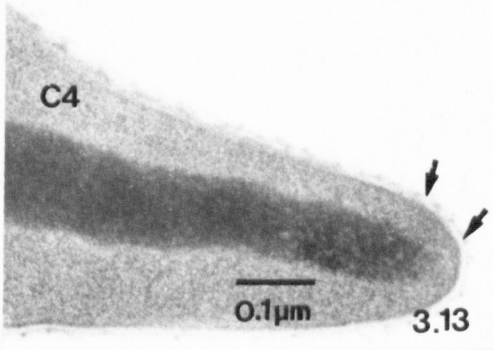
Fig. 3.11: The lateral ala of the third-stage cuticle of Nematospiroides dubius appears fluid-filled (FF). The epicuticle (E) is multilayered (see inset) and the cortical zone (C) is granular. The striated material of the basal zone (B) does not extend across the lateral ala. Below the striated material, an additional fibrous (F) and a granular (G) layer are present. Note the convoluted surface epidermal membrane (→).

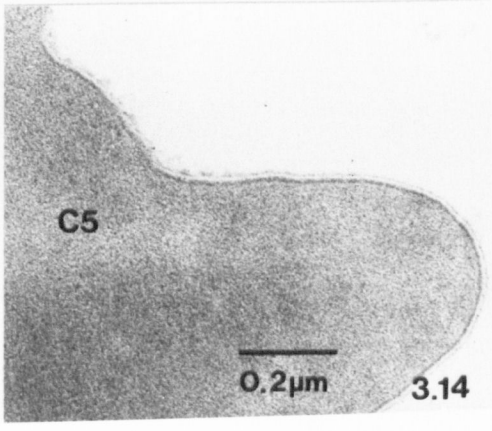
Fig. 3.12: Transverse section through the fourth-stage cuticle (C4) of Nematospiroides dubius. The epicuticle (E) is trilaminate (→). Several layers are visible within the basal zone (B) and a dense bar is present in the longitudinal ridge (R). Hemidesmosomes (H) are present between cuticle and epidermis.

Figs 3.13 and 3.14: Comparison of the surface coat present over the longitudinal ridges of the fourth-stage (C4) and adult (C5) cuticles of Nematospiroides dubius. The surface coat (→) extends across the top of the ridge only in the C4.


E
C
C
0.1µm
E
FF
C
F
G
C
B
0.2µm
3.11


R
C4
E
C4
B
H
H
0.2µm
3.12


C4
0.1µm
3.13


C5
0.2µm
3.14

hemidesmosomes (Fig. 3.7). The epidermal membrane could be seen beneath the cuticle (Fig. 3.7. See also Figs 4.2 and 4.3).

3.3.4 Fourth-stage and adult cuticle

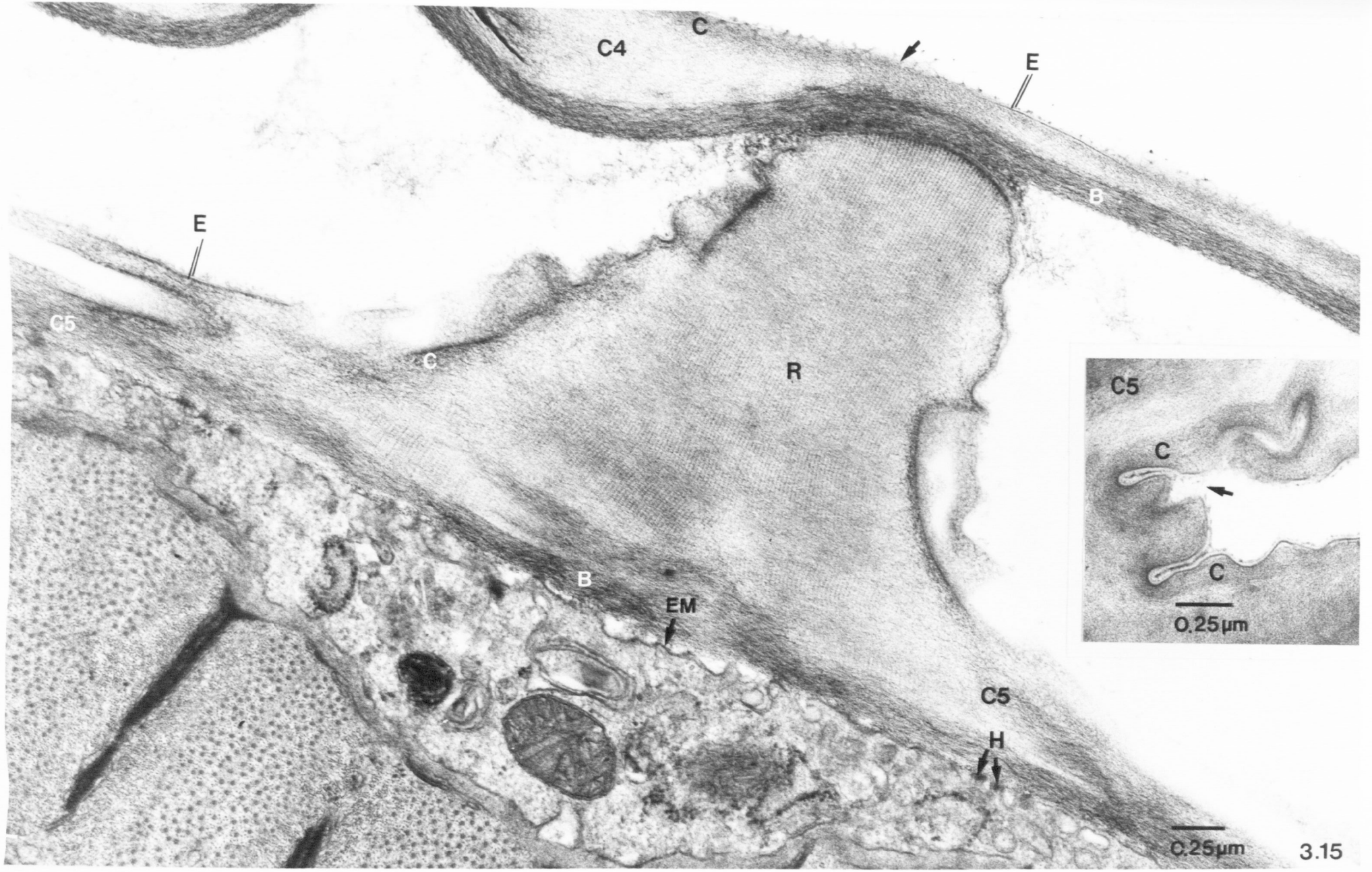
In contrast to the first- and third-stage cuticles, neither the fourth-stage nor the adult cuticle was clearly layered when viewed in transverse section (Figs 3.12 and 3.15). However the basal zone, which consisted of layers of dense fibres, could be distinguished from the granular cortical zone, and the outer cortical zone was often more electron-dense than the rest of the cortical region (Figs 3.12 and 3.15).

Both cuticles were bounded by a trilaminate structure which measured between 13 and 17 nm in thickness. A "felt-like" coat up to 25 nm thick was present on the outer face of the cuticle (Figs 3.12 to 3.15). In the fourth-stage cuticle, the coat extended over the tips of the cuticular spines, but the spines of the adult cuticle were never covered by a "fuzzy" coat (Figs 3.13 and 3.14). Oblique sections showed the surface region of the cuticle to be composed of a meshwork of hexagonal subunits (Fig. 3.15).

Fourth-stage and adult cuticles were characterized by the presence of about 30 longitudinal ridges running along the length of the animal. When viewed in transverse section, each cuticle measured about 2.5 µm in thickness, where the measurement included the ridges, but between ridges, the cuticle was about 0.5 µm thick. Embedded within the ridges of the fourth-stage cuticle were dense bars (Fig. 3.12). In contrast, the median zone of the adult cuticle was less dense and contained a "finger-print" pattern which appeared to be composed of "parallel plates" about 12 nm apart (Fig. 3.15).

Beneath the fourth-stage and adult cuticle, a plasma membrane could be seen bounding the epidermis (Figs 3.12 and 3.15). See also

Fig. 3.15: The adult cuticle (C5) of Nematospiroides dubius just prior to the loss of the fourth-stage cuticle (C4). Both the C4 and C5 are bounded by a trilaminate epicuticle (E) which has a fuzzy coat on its surface. The fibres making up the coat form a hexagonal meshwork (→, see inset). Note the "finger print" pattern present within the longitudinal ridge (R) of the C5. The layers within the C4 and C5 are not distinct but the basal zone (B) and the outer region of the cortical zone (C) appear more dense than the remainder of the cuticle. The epidermal membrane membrane (EM) is visible, and hemidesmosomes (H) are present between the cuticle and epidermis.



3.15

Fig. 3.16: Diagrammatic representation of the different cuticles of Nematospiroides dubius. The first-stage (C1) and third-stage (C3) cuticles are similar, except that the epicuticle of the C3 is multilaminate. The fourth-stage (C4) and adult (C5) cuticles also share a number of features, but are rather different to the other cuticles. The C1, second-stage (C2) cuticle, and C3 exhibit a longitudinal specialization, an ala (LA), along each side, while the C4 and C5 are characterized by a series of longitudinal (R) ridges. Not drawn to scale.

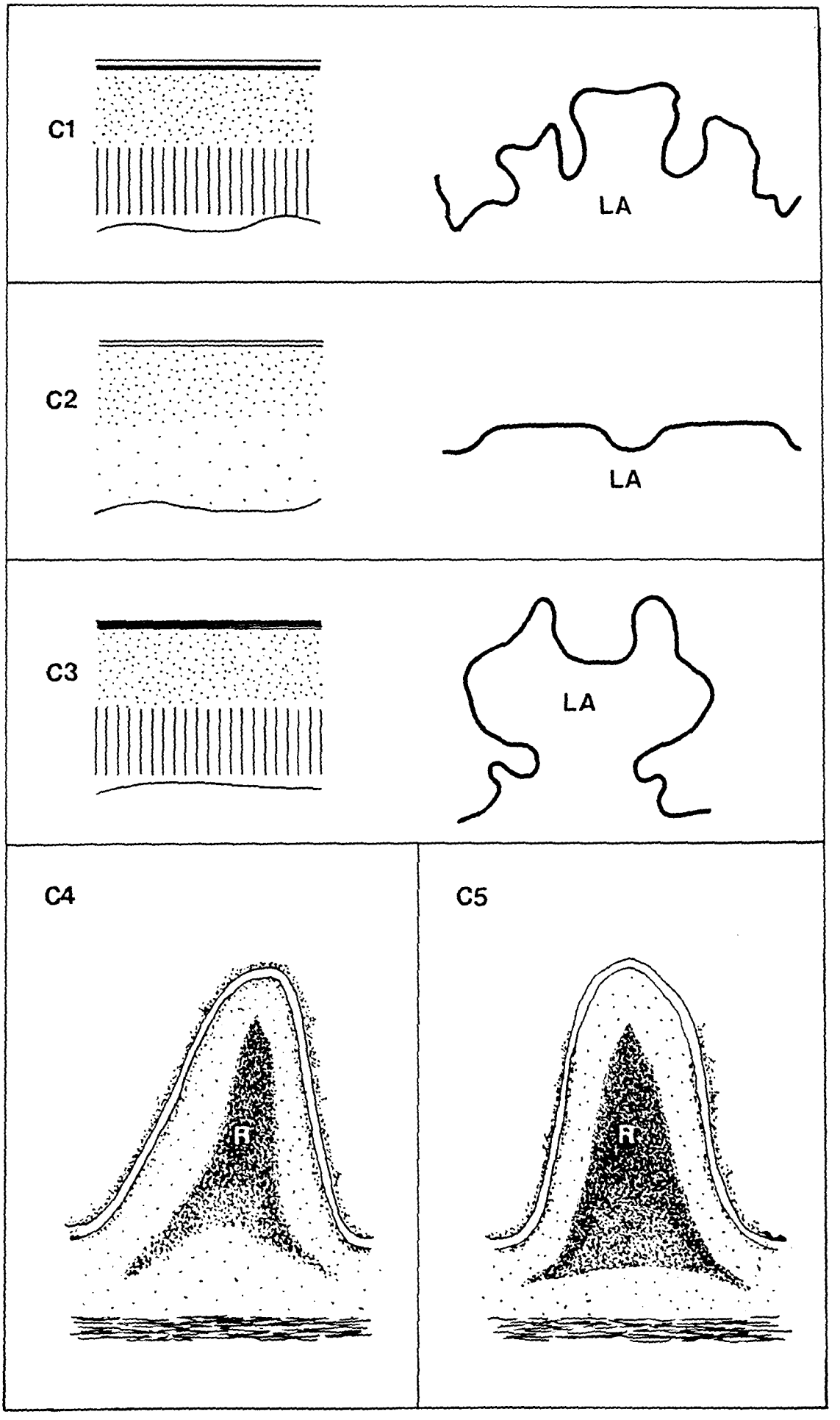


Fig. 3.16

Figs 4.18, 4.22 and 7.11). Hemidesmosomes were apparent where cuticle and epidermis joined (Figs 3.12 and 3.15. See also Figs 4.20 and 7.11).

3.4 DISCUSSION

The parasitic nematode, N. dubius, emerges from this study as an organism capable of diversity of form (Fig. 3.16). At each moult a different cuticle was synthesized suggesting that new sets of genes are "switched on" during development. Thus, N. dubius may be considered to be a polymorphic organism, as defined by Davey (1972), after Wigglesworth (1954). Davey (1972) regards polymorphic development to be a prerequisite for successful parasitism. According to him, "developmental plasticity" is essential for coping with environments that are discontinuous in time and space.

In keeping with this idea, some differences in the structure of the cuticle of N. dubius may be related to the shift to parasitism which occurs during the third-stage of the life cycle. But other differences in the structure of the cuticle show no obvious relationship to the environment of the worm.

Before the relationship between the structure of the cuticle of N. dubius and its environment is discussed, the features common to each cuticle will be mentioned.

3.4.1 Features common to each cuticle

One of the most obvious features of the cuticle of N. dubius is that the cuticle from each stage is stratified into layers or "zones", the outermost of which is always laminate. Comparable zones have been

recognized in the cuticle of most nematodes studied to date (Bird, 1971; 1980; Lee, 1977).

The stratification of the fourth-stage and adult cuticles observed in this study was not as clear as that described by Bonner et al. (1970), particularly within the fourth-stage cuticle. This difference is probably of little significance, however, because it is likely to have been caused by variations in the thickness of sections and in the intensity of staining.

The cuticle of N. dubius is always separated from the underlying epidermis by the epidermal membrane. Although the trilaminate structure of the epidermal membrane could not always be detected in the electron micrographs, it was usually visible with sufficient clarity to establish its identity beyond doubt.

The presence of a membrane bounding the epidermis is well established in some other nematodes such as T. spiralis and N. brasiliensis (Bruce, 1970; Bonner & Weinstein, 1972a. See also Sommerville, 1982). However, no epidermal membrane was identified below the cuticle in M. javanica (Bird & Rogers, 1965). In P. silusiae the epidermal membrane was not always present (Samoiloff & Pasternak, 1969), and Bonner et al. (1970) observed discontinuities in the epidermal membrane of N. dubius. The epidermal region of nematodes is notoriously difficult to fix and it seems likely that inadequate fixation accounts for the discontinuous appearance of the plasme membrane in some instances (Bird, 1971).

A third feature to be shared by each stage of N. dubius is the way in which the cuticle appears to be attached to the underlying epidermis. Dense patches resembling hemidesmosomes appear to play a role in linking cuticle and epidermis. Hemidesmosomes are thought to link epithelial cells with the extracellular connective tissue matrix in

vertebrate tissue (Staehelin, 1974). Similar dense patches have been described between cuticle and epidermis in a number of species of nematode (for example, Dick & Wright, 1973; Hamada & Wertheim, 1978; Lee, 1970; Wisse & Daems, 1968).

It seems implicit in some accounts of the cuticle that hemidesmosomes are the only structure connecting the cuticle to the underlying epidermis (Hamada & Wertheim, 1978; Lee, 1970). This may be so, and no other structures were detected in this study. But fibres have been described in other species of nematode and may be commonly involved in connecting epidermis and cuticle (Bonner et al., 1970; Bonner & Weinstein, 1972a; Bruce, 1970; Kozek, 1971; Wisse & Daems, 1968).

Thus each cuticle of N. dubius is a layered structure which is separated from the underlying epidermis by the epidermal plasma membrane. Hemidesmosomes appear to play a role in linking the cuticle to the epidermis.

The features of the cuticle, described above, are present at each stage of the life cycle of N. dubius and are fundamental to the structure of the animal. Superimposed on these basic similarities, however, are marked ultrastructural differences between the stages.

3.4.2 Ultrastructural differences related to parasitism

It is tempting to correlate the changes which occur in the structure of the cuticle with the environment in which the worm lives and, in particular, with the development of parasitism. The cuticle of fourth-stage larvae and adult worms, which both live in the small intestine of the host, appear similar but differ markedly from those of the free-living stages (Fig. 3.16).

A fundamental difference between the free-living and parasitic stages of N. dubius was the surface morphology of the cuticle. In the fourth-stage and adult worms, the cuticle was thrown into a series of longitudinal ridges which were also descibed by Bonner et al. (1970). These ridges are commonly thought to be important in allowing the worm maintain its position coiled around, and between, the villi of the small intestine of the host (Kleinschuster et al., 1978; Lee & Atkinson, 1977). Similar ridges occur in related genera which live in the same sort of environment, for example Haemonchus, Ostertagia and Cooperia but in genera such as Paralibyostrongylus and Trichostrongylus, the ridges are inconspicuous (Skrjabin, 1952). As far as I know, there is no experimental evidence to support this assertion, and perhaps some care should be taken in ascribing function based on our intrinsic understanding of conditions in the intestine.

In contrast to the longitudinal ridges of the parasitic stages, the cuticle of the free-living stages was smooth apart from two lateral alae. Small free-living nematodes, that live in soil or mud, move on their sides, and the lateral alae are believed to assist in locomotion. During locomotion in these nematodes, an ala will be against the substratum providing a broad area of contact between the cylindrical body of the nematode and the substratum (Lee & Atkinson, 1977).

Apart from this gross morphological difference between the cuticles of free-living and parasitic stages, ultrastructural differences were also noted in the epicuticle. In keeping with observations made by Bonner et al. (1970), fourth-stage larvae and adult N. dubius were found to be bounded by a dense "fuzzy" coat when stained with uranyl acetate and lead citrate. In this study, however, the surface coat was only found over the apex of the ridges in fourth-stage

cuticle, while the tips of the ridges in adult worms appeared smooth. In contrast, no such coat was detected on the surface of free-living stages using these stains. (It should be noted, however, that cationized ferritin binds to the surface of each cuticle except that of infective larvae, and this will be discussed in Chapter 6).

A thick surface coat has also been demonstrated on the surface of other nematodes that parasitize an animal host, using uranyl acetate and lead citrate. A dense felt-like coat was detected on the surface of fourth-stage larvae and adult N. brasiliensis (Bonner & Weinstein, 1972a; Lee, 1970). Similarly the surface of second- and third-stage larvae of A. suum was bounded by a dense fuzzy layer (Morseth & Soulsby, 1969), as was the surface of M. muris (Hamada & Wertheim, 1978) and T. suis (Jenkins, 1969).

The origin of this surface coat is of particular interest because it forms part of the host-parasite interface. It is unlikely that the surface coat is of host origin in N. dubius because it was present on the surface of the adult cuticle before the fourth-stage cuticle had been shed (Fig. 3.15).

Rather it seems probable that the surface coat forms as an intrinsic part of the new cuticle, either from newly synthesized molecules, or from breakdown products of the previous cuticle (see Chapter 4 for an account of the formation of the fourth-stage cuticle). Certainly the inner layers of the third- and fourth-stage cuticles break down during cuticle formation (Fig. 3.15 & Figs 4.12 to 4.20). One way of determining whether the precursors of the epicuticle are synthesized at each moult or derived from the preceding cuticle, would be to trace the progress of precursor molecules that had been previously labelled.

In contrast to the parasitic stages of N. dubius, no surface coat was detected on the free-living stages using uranyl acetate and lead

citrate. Differences in the ultrastructure of the surface region can also be detected between the infective- and parasitic stages of N. brasiliensis (Lee, 1965; 1966; 1970). From the published electron micrographs, it appears that the surface of the third-stage cuticle of N. brasiliensis is smooth, but the fourth-stage and adult cuticles are bounded by a dense coat of filaments (Jamuar, 1966; Lee, 1965; 1966; 1970).

The epicuticle of the third-stage cuticle is multilaminate and so differs from that of all the other stages which are trilaminate. The third-stage larva is also exceptional in that it is an infective- stage in which development is halted until a suitable host is located. It seems possible that a multilaminate epicuticle may be related to an increased impermeability. Certainly the epicuticle of a number of species of nematode, particularly of the resistant stages, is multilaminate when viewed in transverse section (Despommier, 1975; Smith, 1970; Wharton et al., 1981). But the surface of some adults may also be multilayered (Baldwin & Hirschman, 1975; Dick & Wright, 1973; Wright, 1968).

Thus one explanation for the differences in the structure of the cuticle of N. dubius lies in the transition to parasitism. But to attribute these ultrastructural differences solely to environmental influences is too simplistic an explanation. A closer examination reveals differences in the structure of the cuticle which do not appear to be related to the environment.

3.4.3 Ultrastructural differences not related to the environment

If the environment was the major determinant of the structure of the cuticle of N. dubius, one would expect the cuticle of the first-,

second- and third-stage larvae to be similar because these worms are all free-living (Fig. 3.16). Indeed the first- and third-stage cuticles are almost identical. The fine detail of the epicuticle differs, and the third-stage cuticle is thicker that that of the first-stage, but the fundamental pattern of the two cuticles is similar.

Although the second-stage larva of N. dubius occupies what apparently is an identical ecological niche to the first- and infective-stage worms, the second-stage cuticle is markedly different from the first- and third-stage cuticles. Unlike the other two free-living cuticles, the epicuticle of the second-stage cuticle is thinner than that of other stages. Moreover, although second- and first-stage larvae occupy the same ecological niche, only the second-stage cuticle appears to attract E. coli to its surface (Figs 6.54 to 6.56).

The second-stage cuticle further differs from that of first- and infective-stage larvae in that it contains no striated material in the basal zone. Using freeze-fracture techniques (Figs 3.8 to 3.10) and oblique sections (Fig. 7.22), the striated material of the infective-stage larvae, at least, appears similar to that described for other species of nematode (Bird & Buttrose, 1974; Popham & Webster, 1978). The nature and function of this material is unknown, but several suggestions have been put forward. For example, the striated material is thought to provide the tensile strength of the cuticle which is necessary for movement (Nicholas, 1984; Wright, 1968). Alternatively, Samoiloff (1973) proposed that it might be involved in cuticular transport. The role of the striated material in movement will be considered further in Chapter 7.

The development of the second-stage cuticle into the sheath was not followed closely in this study, but it would be an interesting exercise to do so. The second-stage cuticle appeared to increase in

thickness prior to the partial moult because the sheath was about twice the thickness of the cuticle when still attached to the epidermis. In comparison, during the third and fourth moults, the old cuticle was always thinner after it had separated from the epidermis.

As already implied, it is tempting to categorize the third-stage cuticle as "free-living". However, it is the third-stage cuticle that first faces the host for any length of time. Indeed, the third moult only occurs 48 to 72 hours after infection so that the third-stage cuticle is exposed to the host for about the same length of time as the fourth-stage cuticle. Why then is the third-stage cuticle similar to that of first-stage larvae but different to that of fourth-stage worms ? One must assume that following infection some qualities of the third-stage cuticle, particularly those relating to permeability, are markedly different from those present prior to infection. Perhaps in N. dubius, the sheath offers protection while the properties of the third-stage cuticle change to become more like those present in fourth-stage larvae and adult worms.

That the first- and third-stage cuticles of N. dubius are similar is also curious from another point of view. Prior to infection, first- and third-stage larvae occupy similar habitats, but the third-stage cuticle is separated from the environment by the second-stage cuticle. [This cuticle or sheath is commonly called "protective" but its function is not clear. Certainly Ellenby (1968) showed that the sheath reduced water loss in Haemonchus contortus]. So although the two cuticles appear similar and the two larvae occupy a similar niche, the third-stage cuticle may not be in direct contact with the environment.

Thus the ultrastructure of the cuticle of N. dubius alters at each moult. Fundamental differences in the structure of the cuticle occur between the free-living and parasitic stages (see Section 3.4.2). But

it is not always possible to relate the structure of the cuticle of the free-living stages to the environment in which these worms live. Until more is known about the composition of the cuticle, the implications of these ultrastructural features to the animal will remain largely a matter for speculation.

CHAPTER 4

FORMATION OF THE FOURTH-STAGE CUTICLE OF N. dubius

4.1 INTRODUCTION

Whether or not the epicuticle of the nematode cuticle is a plasma membrane may well be regarded as a pedantic question by those who consider the function of a structure as all important. However, as Martinez-Palomo (1978) pointed out, "the issue is of more than semantic interest; it is of great importance for the understanding of host-parasite interactions in nematode infections".

To decide whether or not the epicuticle of the nematode cuticle is a plasma membrane, one needs to know how and where the epicuticle forms during the synthesis of the cuticle. Of course, if during cuticle formation the newly formed trilaminate structure that characterizes the epicuticle appeared below the epidermal membrane, then the latter would become the new epicuticle on the surface of the animal, while the new structure would become the plasma membrane of the epidermis. In this way, the epicuticle would represent a modified plasma membrane. On the other hand, if the newly formed trilaminate structure appeared outside the epidermal membrane, then it must be extracellular. In this case, at no time during the formation of the cuticle could the epicuticle be considered a plasma membrane.

Because there are very few ultrastructural studies of cuticle formation, there is still doubt as to how and where the epicuticle forms. Moreover interpretation of studies of this nature pose several problems. Firstly, micrographs are a static record of a series of past events which means the observer must decide the order of a given

chain of events. Secondly, cuticle development is not always related to the age of the worm (Samoiloff & Pasternak, 1969; Davey, 1965; Bonner & Weinstein, 1972a). Indeed, the stage of development may vary even along the length of one worm (Samoiloff & Pasternak, 1969). Thirdly, there are major technical difficulties in adequately fixing the epidermal region of nematodes which is notoriously inaccessible to chemicals. As Bird (1971) noted, the external surface of the epidermis, below the relatively impermeable cuticle, is one of the most difficult to fix and stain.

Technical difficulties can be overcome, of course, but the problem posed by the first two points is more complex. There are obvious difficulties in reconstructing a sequence of events from a group of micrographs, and not knowing the stage of development of the worm intensifies the difficulty. One way to overcome the problem is to define a set of criteria which a sequence of micrographs must satisfy before they can be accepted as evidence for an event. Thus, although one micrograph alone may not be convincing, a series of micrographs might adequately describe a chain of events.

Ultrastructural evidence required to decide where the epicuticle forms must, at the very least, include micrographs that depict the following events.

1. The intact epidermal membrane prior to apolysis, that is, before the old cuticle and epidermis separate. Even if the trilaminate structure of the epidermal membrane cannot be recognized, the epidermal membrane can often be identified by characteristic dense regions which resemble hemidesmosomes.

2. Precursor elements must be shown either above or below the epidermal membrane which should still be discernible as a continuous dense line.

3. The secreted precursor elements, either above or below the epidermal membrane, should show differentiation.

Two ultrastructural studies fulfill these requirements. Bonner & Weinstein (1972a) studied the early stages of cuticle formation in the third moult of N. brasiliensis and in the final moult of N. dubius, while Martinez-Palomo (1978) examined the genesis of the first cuticle of the microfilaria of O. volvulus. Both studies concluded that the epicuticle differentiated by a layering of fibrillar components secreted on the outside of the epidermis.

The sequence of micrographs presented by Bonner & Weinstein (1972a) is particularly convincing. It is of interest to note that only when the precursor elements forming the new cuticle measured about 100 nm in thickness, did the outer fringes differentiate into a layered structure that later became the epicuticle. The epidermal membrane and epicuticle were never juxtapositioned. Similarly, the series of micrographs presented by Martinez-Palomo (1978) showed that while the epidermal membrane, recognizable by the small dense regions that occurred along its length, was discernible throughout cuticle formation, differentiation of the fibrillar elements outside the epidermal membrane was only present late in the series of micrographs. Accordingly, in both cases the sequence of micrographs taken as a whole strongly suggests that the epicuticle forms outside the epidermal membrane.

Further support for the notion that the epicuticle is extracellular comes from a study of the last moult of S. obvelata (Dick & Wright, 1973). Although these workers did not set out to study the early events of cuticle formation, they presented a micrograph which achieves this result (Fig. 18, Dick & Wright, 1973). The micrograph shows that the precursors of the new cuticle, outside the epidermal

membrane, were undifferentiated except for two small regions in which layers were distinct. The epidermal membrane was obviously intact before the new epicuticle was complete.

Therefore evidence from four species of nematode suggests that the epicuticle is formed outside the epidermal membrane. In these species, the epicuticle fits the definition used by Locke (1982) to describe the term "envelope".

Evidence against the notion that the epicuticle forms outside the epidermal membrane is scant. Perhaps the most outstanding study is that of Lee (1970) who studied the last moult of N. brasiliensis. Although he stated that the new "membrane" formed beneath the epidermal membrane and showed the two trilaminate structures already present, he did not include a micrograph of the earlier events. One must ask; which membrane came first ? In short, this series of micrographs does not have the continuity of those of Bonner & Weinstein (1972a) and Martinez-Palomo (1978).

Similarly, Bird (1977) studied the formation of the first- and second-stage cuticles of M. javanica. Although the events before and after cuticle formation were well illustrated, the micrographs do not demonstrate where the epicuticle first appeared. On the other hand, Johnson, Van Gundy & Thomson, (1970) simply concluded that the trilaminate osmiophilic zone formed at the edge of the epidermis but offered no evidence for earlier events.

Although the ultrastructural evidence indicates that the epicuticle forms outside the epidermal membrane, particularly when viewed in light of the criteria presented earlier, some authors remain sceptical (Bird, 1984). However, because it is fundamental to my discussion about the nature of the epicuticle to establish beyond doubt

whether the epicuticle is an extracellular envelope, I examined the way in which the fourth-stage cuticle of N. dubius formed.

4.2 MATERIALS AND METHODS

The materials and methods used in this section have been described (see Sections 2.2 and 2.3). To study the formation of the fourth-stage cuticle, worms were removed from mice 48, 72, 83, 96 and 120 hours after infection. Some larvae removed from mice 83 hours after infection were labelled with cationized ferritin (see Section 7.2).

4.3 RESULTS

The events taking place during the formation of the fourth-stage cuticle of N. dubius were established and have been summarized in Fig. 4.1. These results will be discussed later.

Although the process of cuticle formation could be closely followed, it was not possible to relate cuticle formation to the age of the worm because the rate at which third-stage larvae of N. dubius developed within the host varied among individuals. An extreme example of individual variation is demonstrated by comparing Fig. 4.6 with Fig. 4.19. In the former, the worm was removed from the host 83 hours after infection and the fourth-stage cuticle was not apparent. On the other hand, Fig. 4.19 is from a worm removed from the host only 72 hours after infection, yet the formation of the fourth-stage cuticle was well advanced. The micrographs illustrating the different stages of cuticle formation presented here were taken from at least 15 individual worms

Figs 4.1 (a to f): Diagrams depicting the formation of the fourth-stage cuticle (C4) of Nematospiroides dubius. basal zone, b; cortical zone, c; epicuticle, ec; epidermis, e; epidermal membrane, em; third-stage cuticle, C3; fourth-stage cuticle, C4.

Fig. 4.1(a): Prior to apolysis, the epidermal membrane appears highly convoluted beneath the C3 which is bounded by a multilaminate epicuticle (see Figs 4.2 and 4.3).

Fig. 4.1(b): The first discernible event in cuticle formation is a straightening of the epidermal membrane which is then visible as a trilaminate structure (see Figs 4.4 and 4.5).

Fig. 4.1(c): The connection between the C3 and epidermal membrane is more tenuous as filamentous material accumulates along the outer edge of the epidermis. Vesicles (V) appear to fuse with the epidermal membrane (see Figs 4.6 to 4.12).

Fig. 4.1(d): When the filamentous material between the epidermal membrane and the C3 is between 80 and 130 nm thick, the outer 45 to 75 nm is more electron dense than the inner region. In some areas, the outer region appears layered (see Figs 4.13 to 4.15).

Fig. 4.1(e): The fourth-stage epicuticle is distinct when the new C4 measures between 300 and 500 nm in thickness (see Figs 4.16 to 4.18).

Fig. 4.1(f): Before the C3 is lost, the C4 increases in thickness as more material is added to it (see Figs 4.19 and 4.20).

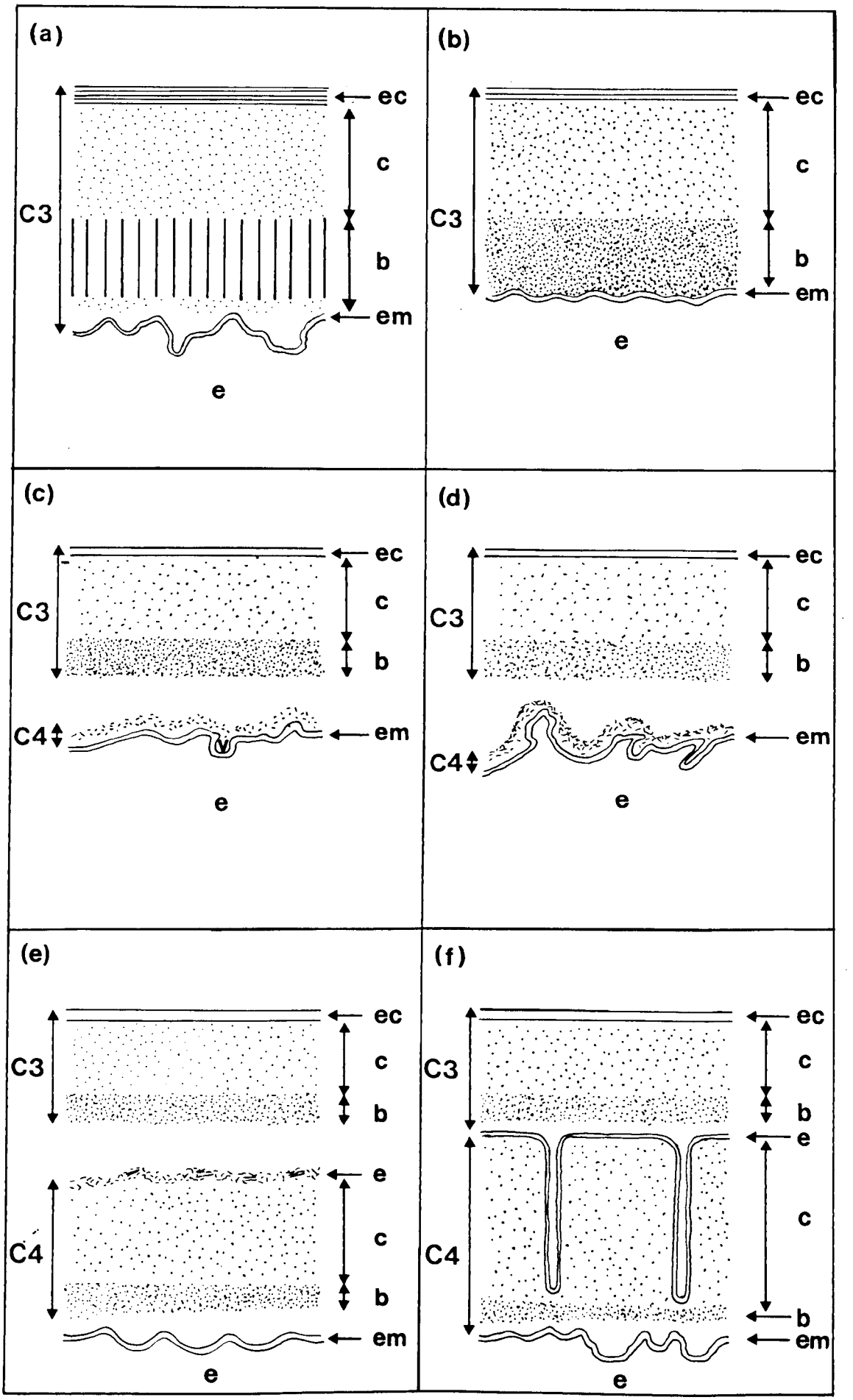

(a)
ec
c
C3
b
em
e
(b)
ec
c
C3
b
em
e
(c)
ec
c
C3
b
C4
em
e
(d)
ec
c
C3
b
C4
em
e
(e)
ec
c
C3
b
e
c
C4
b
em
e
(f)
ec
c
C3
b
e
c
C4
b
em
e

Fig. 4.1

that had been removed from the host between 48 and 96 hours after infection.

As will be noted, most micrographs used in this study of cuticle formation depict events occurring in the lateral epidermal region of the worm. This region was selected because the distinctive lateral alae make it easy to decide whether or not the section was truly transverse, and because a great deal of activity associated with the synthesis of the new cuticle takes place in the epidermal chords.

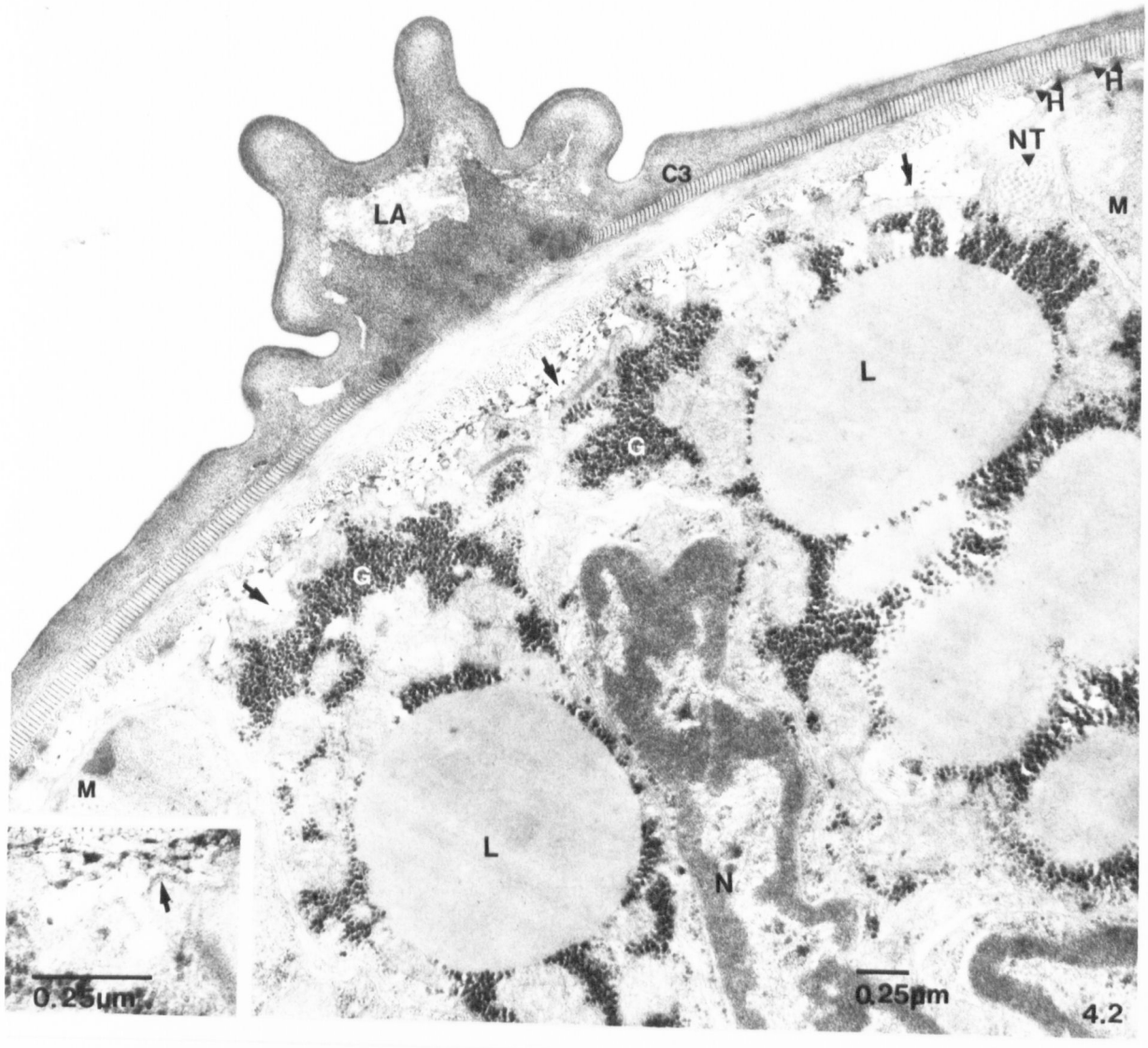
Prior to infection, the lateral epidermal region of third-stage larvae of N. dubius appeared inactive (Fig. 4.2). The nuclei, for example, contained highly condensed chromatin. In addition, large deposits of lipid and glycogen were obvious.

The epidermal surface was highly convoluted. The structure of the epidermal membrane was not distinct as a rule, but there were small regions where the trilaminate structure characteristic of cell membranes could be seen (Figs 4.2 and 4.3). The epidermal membrane in infective larvae was between 7.5 and 10.0 nm thick, while the epicuticle of the third-stage cuticle measured between 20 and 27 nm in thickness. A few hemidesmosomes were apparent in the lateral epidermal region, and they were abundant along the epidermal membrane present over the muscle cells (Fig. 4.3).

After infection of the host, the first discernible event in cuticle formation was a "straightening" of the epidermal membrane (compare Figs 4.4 and 4.5 with Figs 4.2 and 4.3). At this stage, the straightened epidermal membrane could be readily identified as a triple-layered structure which measured between 6.5 and 8.0 nm in thickness (Figs 4.5 to 4.9). Correlated with the straightening of the epidermal membrane was an increase in the diameter of the worms (see Figs 7.5 and 7.6).

Fig. 4.2: The lateral epidermal region of an infective larva of Nematospiroides dubius showing a lateral ala (LA) of the third-stage cuticle (C3). The epidermal membrane (→) is highly convoluted and in some regions appears trilaminate (see inset). Also present are large deposits of lipid (L) and glycogen (G). Nuclei (N) contain condensed chromatin and hemidesmosomes (H) are apparent over the muscle (M) cells. Nerve tissue (NT) is present between the muscle cell and lateral epidermal chord.

Fig. 4.3: The sites of attachment between the third-stage cuticle (C3) and epidermal membrane of Nematospiroides dubius are shown. Note the hemidesmosomes (H) and the epidermal membrane which appears trilaminate (→) in some regions.


LA
C3
H
H
NT
M
L
G
G
M
L
N
0.25µm
0.25µm
4.2

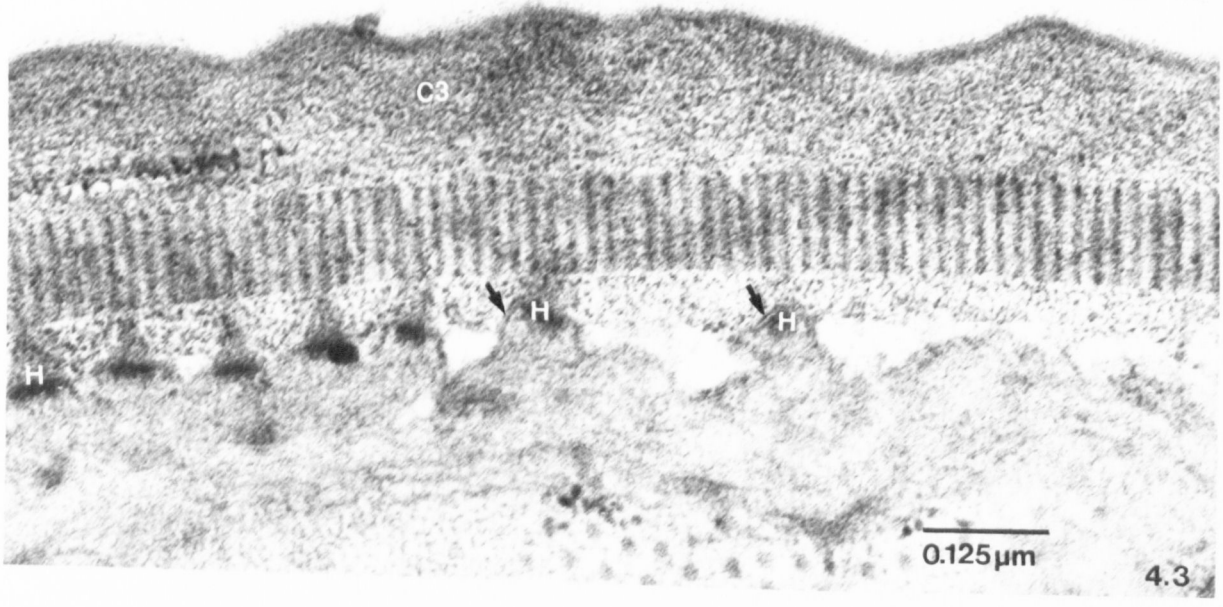
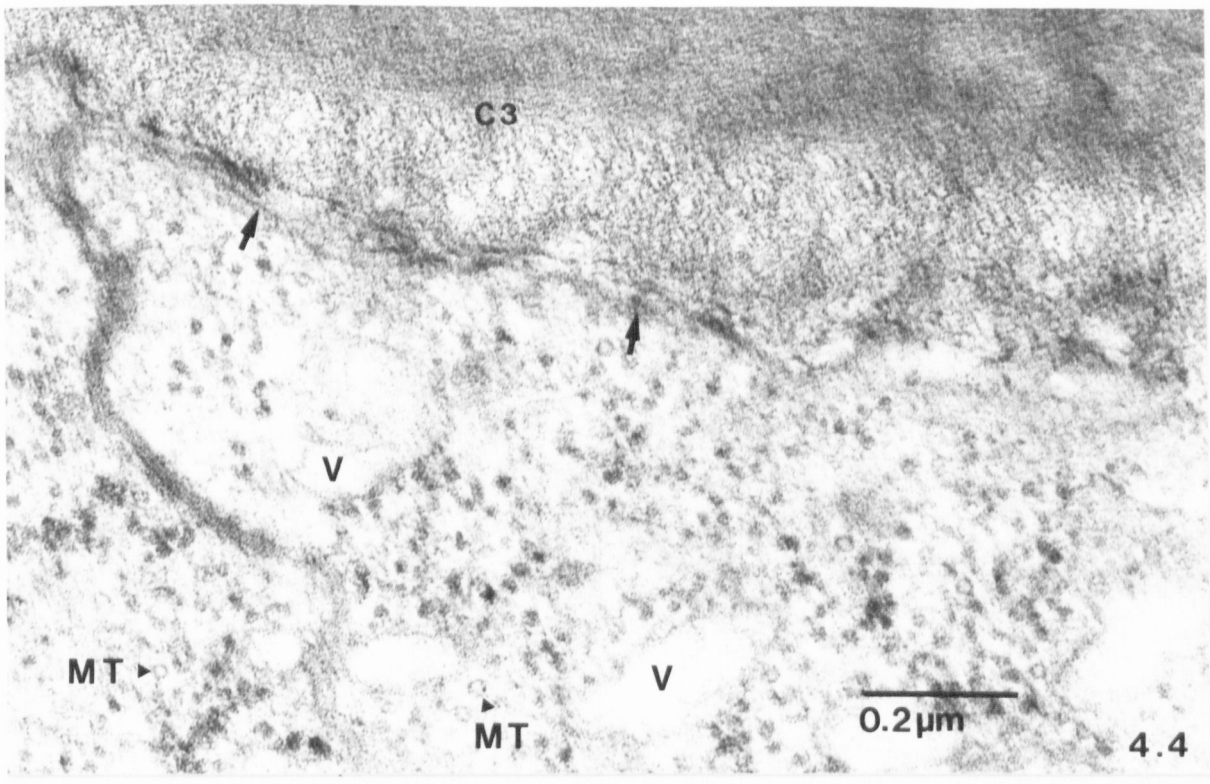
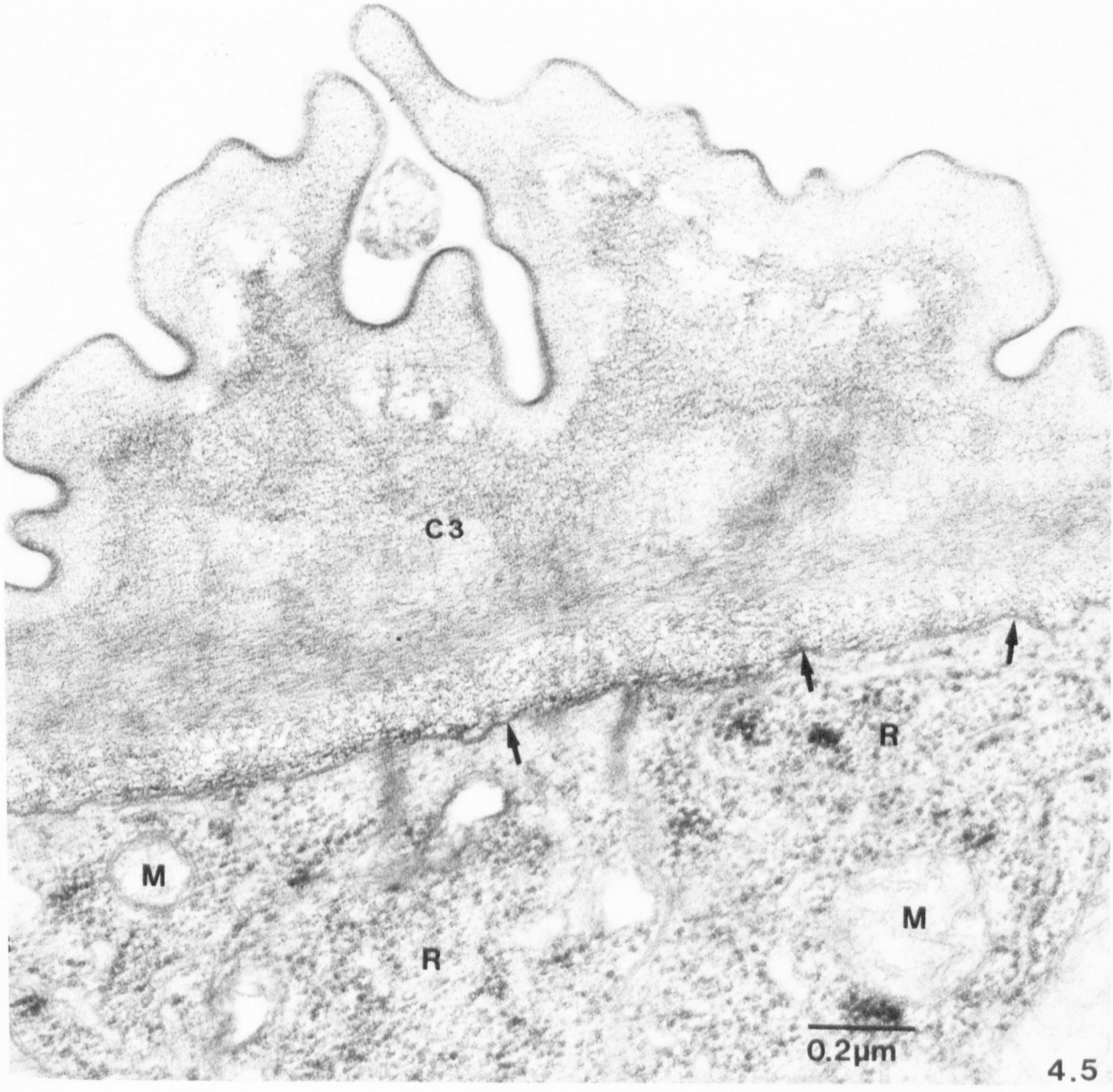

C3
H
H
H
0.125µm
4.3

Fig. 4.4: The lateral epidermal region of the third-stage larva of Nematospiroides dubius prior to the formation of the fourth-stage cuticle. The epidermal membrane (→) is still closely applied to the third-stage cuticle (C3). Vesicles (V) and microtubules (MT) can be seen within the epidermis.

Fig. 4.5: The straightened epidermal membrane of a third-stage larva of Nematospiroides dubius just prior to apolysis displaying the characteristic trilaminate structure (→). The epidermal membrane is closely applied to the overlying cuticle (C3) and the epidermal region contains numerous mitochondria (M) and ribosomes (R).

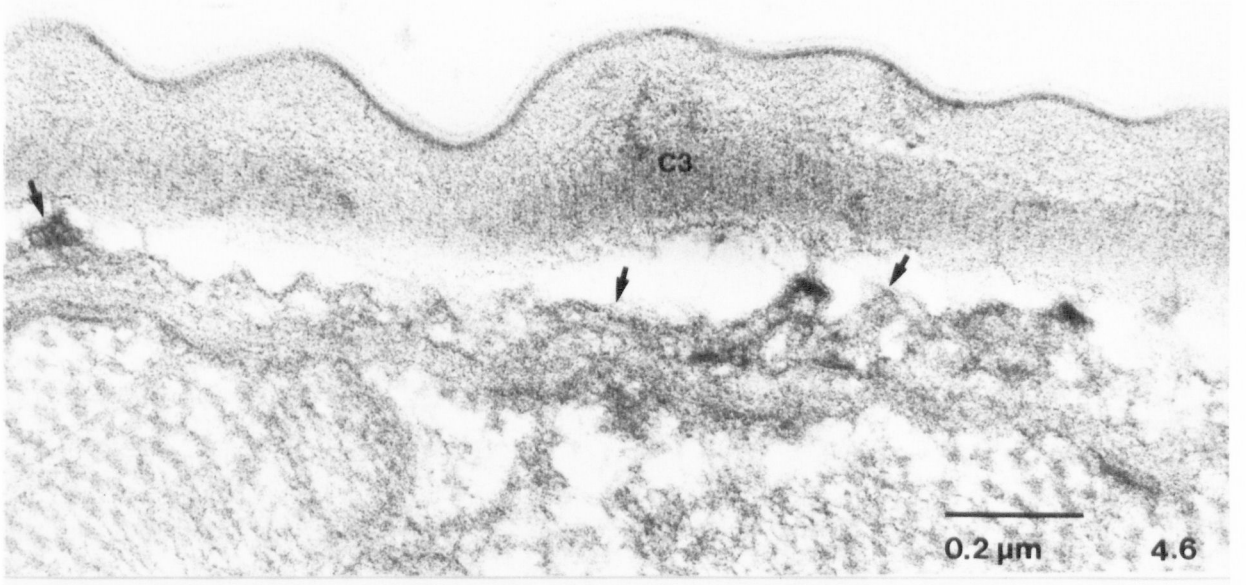


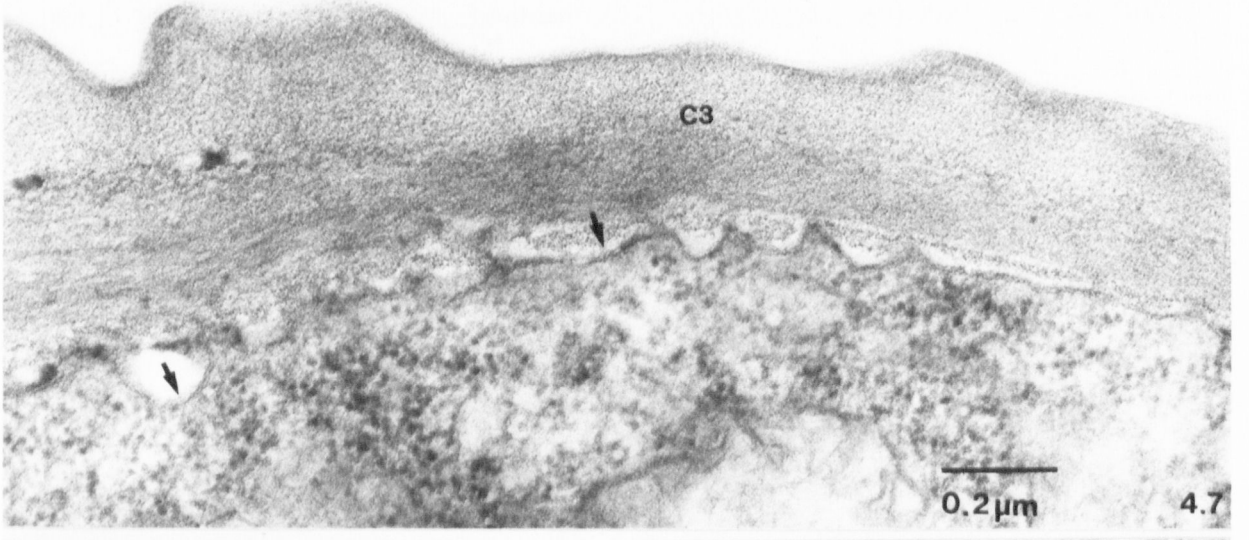
4.4

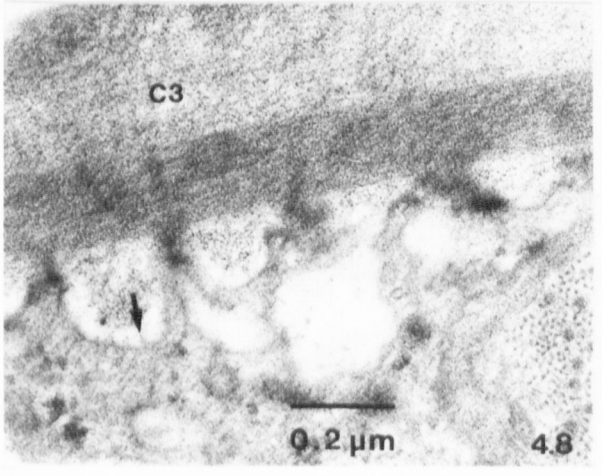


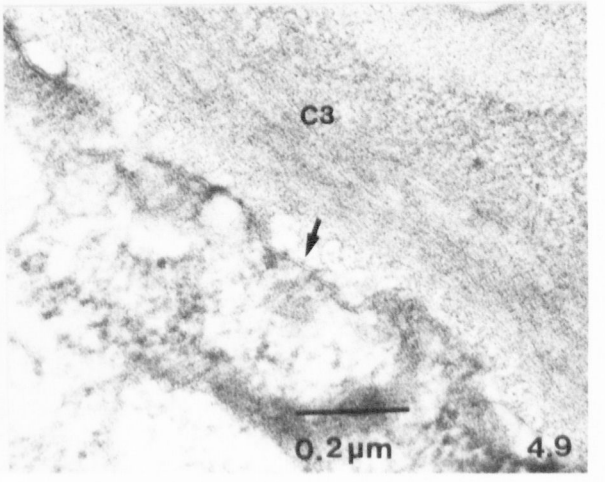
4.5

Figs 4.6 to 4.9: Micrographs showing the epidermal membrane of Nematospiroides dubius just prior to the appearance of the fourth-stage cuticle. Note the trilaminate structure of the epidermal membrane which has fine filaments extending from the outer face (→). Sites of attachment between the third-stage cuticle (C3) and the epidermal membrane appear diminished.


C3
0.2 µm
4.6


C3
0.2 µm
4.7


C3
0.2 µm
4.8


C3
0.2 µm
4.9

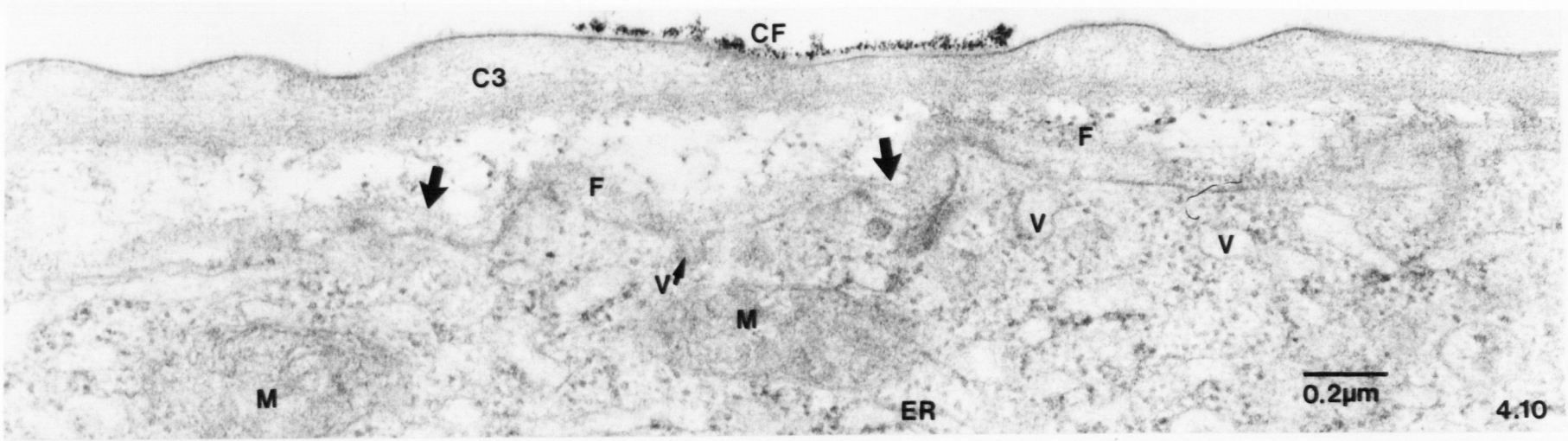
As the epidermal membrane straightened, the hemidesmosomes became less distinct (Figs 4.6 and 4.8), and the connection between epidermis and cuticle more tenuous. Fine filaments, measuring up to 25 nm long, extended from the outer edge of the epidermal membrane (Figs 4.6 to 4.9). In these worms, the epidermal region appeared to be more active than in the infective larvae because mitochondria, ribosomes, vesicles and microtubules were abundant (Figs 4.4 and 4.5).

The precise way in which the epidermis and cuticle separated is unknown. Following apolysis, however, the epidermal membrane was thrown into a series of folds or "plicae". A layer of diffuse filamentous material 80 to 130 nm thick was associated with the outer face of the epidermal membrane (Figs 4.10 to 4.14). Small areas of the outer 45 to 75 nm of the filamentous material was more electron-dense than the inner region lying immediately above the epidermal membrane (for example, see Fig. 4.13). It should be noted that the outer, dense fringe of this filamentous material could be easily distinguished from the epidermal membrane because it was less well defined (Figs 4.11 to 4.14).

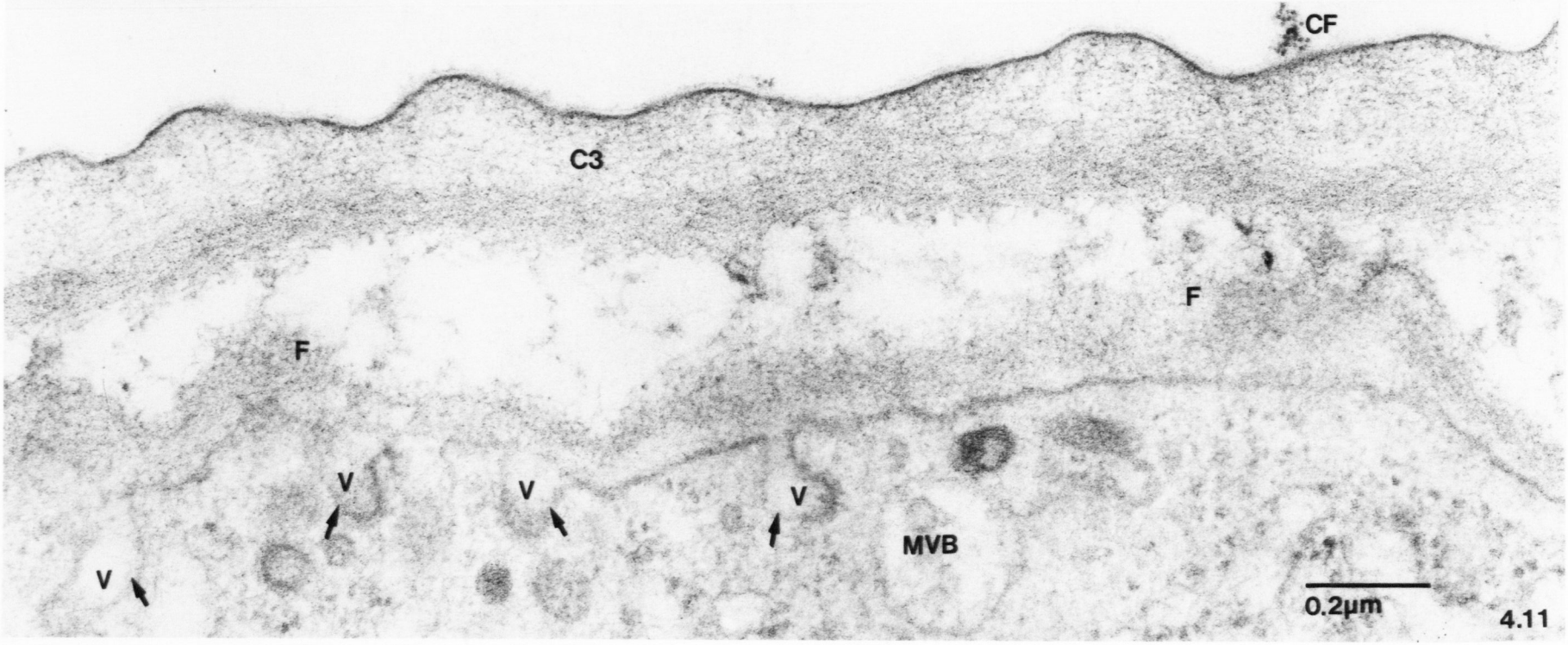
When the filamentous material making up the new fourth-stage cuticle measured between 150 and 300 nm thick, the outer dense region was composed of three layers (Fig. 4.15). The outermost layer of the developing cuticle consisted of a dense mat of filaments up to 30 nm wide. Beneath this dense layer was an electron lucent zone about 7.5 to 9.0 nm thick, below which lay an inner dense region about 25 to 30 nm thick. In addition, in small regions of these outer layers, a distinct trilaminate structure could be discerned (Fig. 4.15).

At this very early stage of cuticle formation, the lateral epidermis contained many vesicles, some of which appeared to fuse with the epidermal membrane (Figs 4.10 to 4.15). Where a vesicle was continuous with the epidermal membrane, its contents were, in effect, on

Figs 4.10 to 4.13: Early events in the formation of the fourth-stage cuticle of Nematospiroides dubius are shown. The third-stage cuticle (C3) has lifted from the underlying epidermis which contains numerous vesicles (V), some of which appear to be fused with the epidermal membrane (→). Also present are multivesicular bodies (MVB), mitochondria (M), ribosomes (R) and endoplasmic reticulum (ER). The outermost region of the filamentous material (F) lying between the C3 and epidermis is electron-dense compared with the inner region, and appears layered in some areas (➡). Clusters of cationized ferritin (CF) molecules can be seen sticking to the surface of the C3.

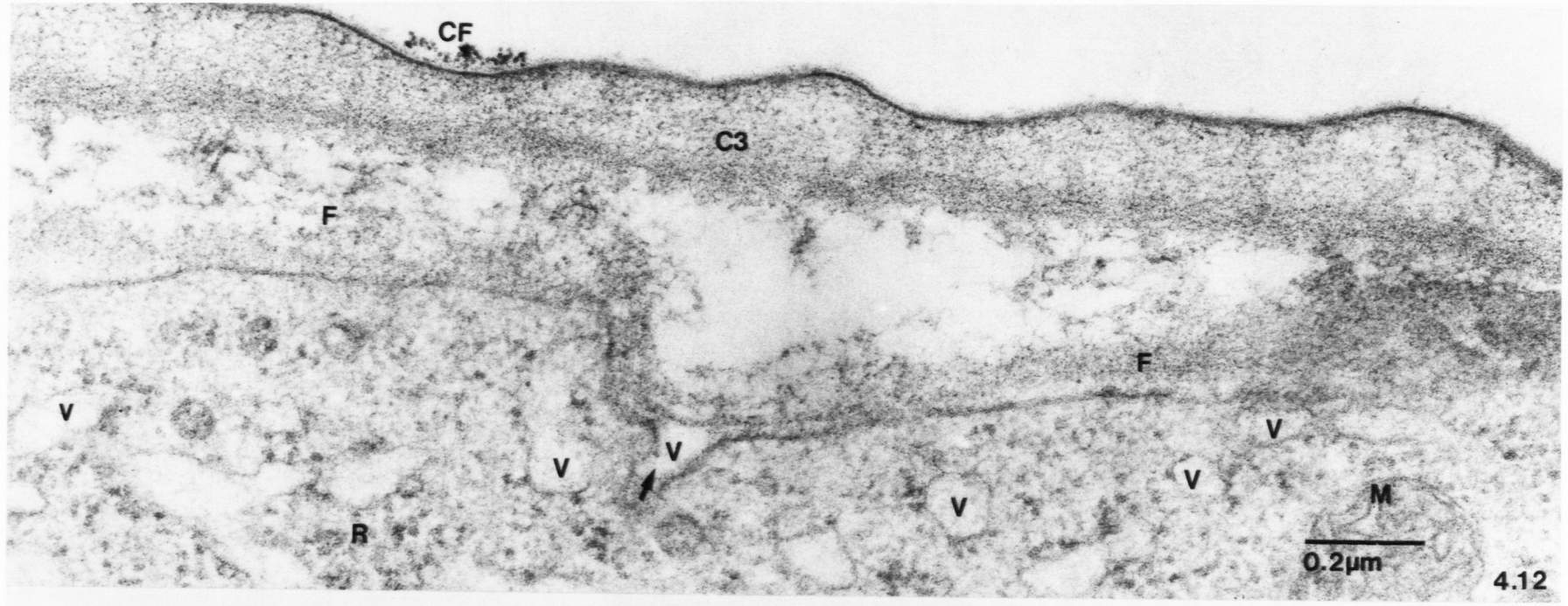


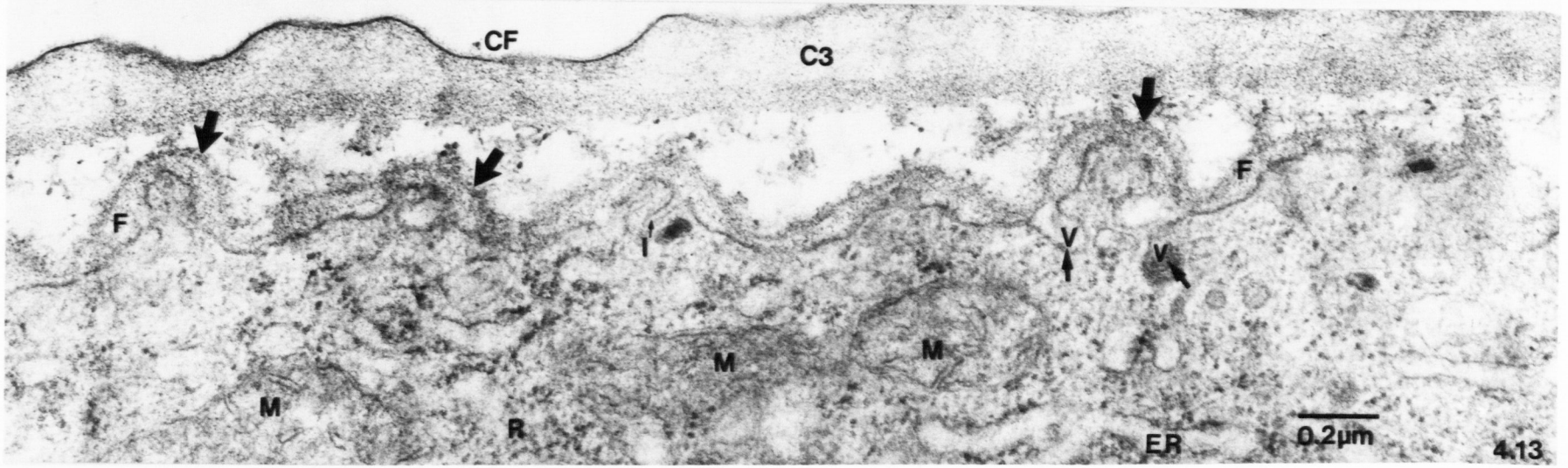
4.10



4.11

Fig. 4.13: Note the epidermal membrane is infolded (I).


CF
C3
F
F
V
V
V
V
V
V
V
M
R
0.2µm
4.12


CF
C3
F
F
V
V
M
M
M
R
ER
0.2µm
4.13

the outer face of the epidermis. Indeed, the filamentous material lying between epidermis and cuticle appeared similar to the contents of some of the vesicles (Fig. 4.11). The epidermal region also contained numerous mitochondria and extensive rough endoplasmic reticulum (Figs 4.10 to 4.15). Multivesicular bodies were sometimes seen (Fig. 4.11).

When the fourth-stage cuticle was between 300 and 500 nm thick, its outer edge was clearly structured (Figs 4.16 to 4.18). Glimpses of a trilaminate stucture could be seen in the electron-dense outer region of the forming cuticle. This trilaminate structure, that is, the epicuticle of the fourth-stage cuticle, measured between 11 and 13 nm in thickness, while the epidermal membrane from the same worm was found to be between 6.5 and 8.5 nm wide.

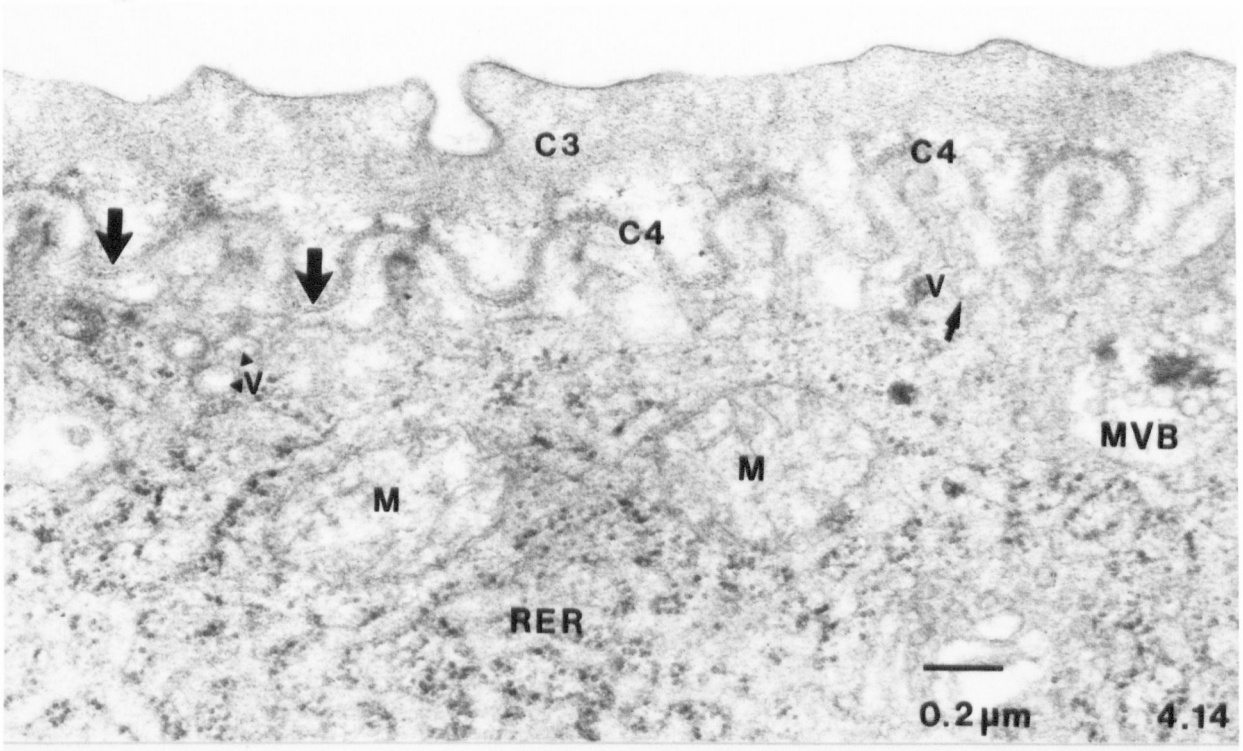
Beneath the epicuticle was a dense layer of filamentous material measuring about 35 nm in thickness. The remaining bulk of the cuticle, however, consisted of several diffuse filamentous layers with a further granular deposit sometimes present on the surface of the epidermal membrane (Figs 4.16 to 4.18).

Oblique sections of the developing cuticle provided insight into the structure of the cuticle (Fig. 4.16). When viewed from one direction, part of the cuticle appeared to be composed of "honeycomb" subunits. However, when viewed from a different angle, the cuticle appeared to be composed of a series of parallel electron-dense lines, 4 to 6 nm wide and 13 to 16 nm apart (Fig. 4.16).

At this stage of cuticle formation, the epidermis still contained many mitochondria (Figs 4.16 to 4.18). Ribosomes were present in the cytoplasm or associated with the membranes of the cisternae and Golgi complexes could be recognized. Numerous vesicles, some of which resembled coated vesicles, were also present. In addition, there were

Fig. 4.14: The epidermal membrane of Nematospiroides dubius is thrown into folds or "plicae" and is topped by the new cuticle (C4) which appears layered (➡). Vesicles (V) are present close to, and are sometimes fused (→) with, the epidermal membrane. Also present is a multivesicular body (MVB), mitochondria (M) and rough endoplasmic reticulum (RER).

Fig. 4.15: The fourth-stage cuticle (C4) shows differentiation at the outermost edge. Glimpses of the new trilaminate epicuticle can be seen within the electron-dense outer region of the developing C4 (circled - see inset). Hemidesmosomes (H) are present between the new cuticle and epidermis. The epidermal region contains rough endoplasmic reticulum (RER) and mitochondria (M). Numerous vesicles (V), including what appear to be coated vesicles (CV) are also present. Note the vesicle fusing with the epidermal membrane (→) and the dense patch (P) present on the epidermal membrane.


C3
C4
C4
V
V
MVB
M
M
RER
0.2 µm
4.14

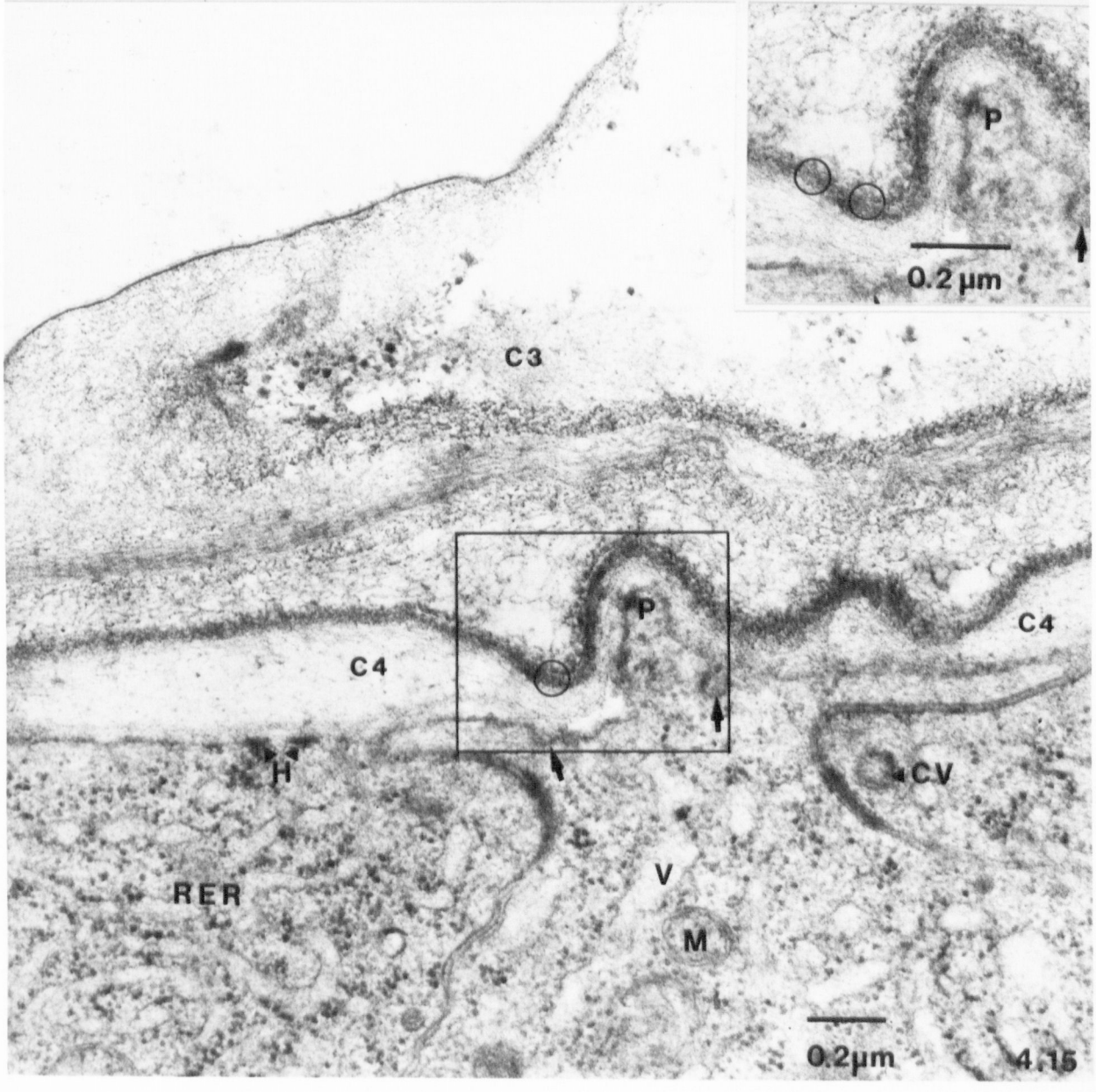
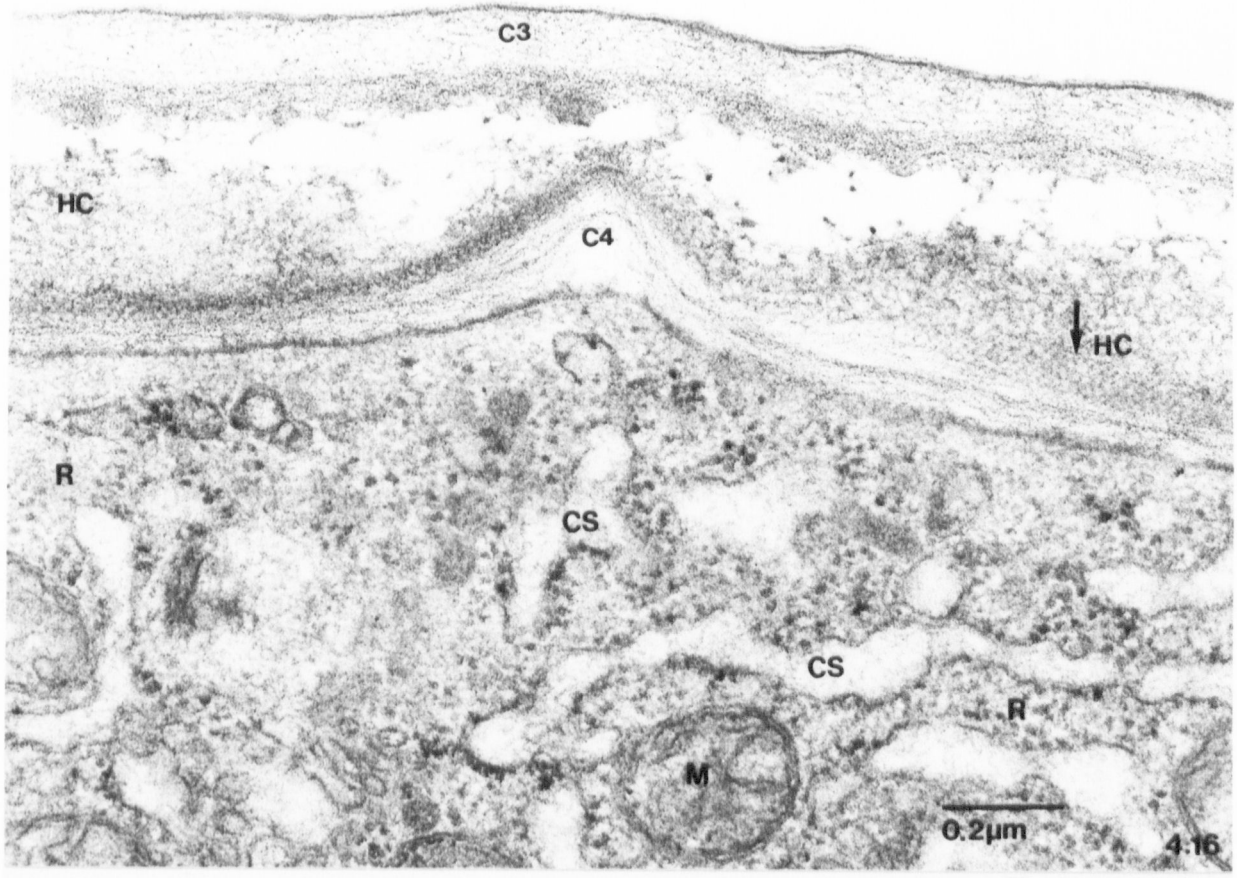

P
0.2 µm
C3
P
C4
C4
H
CV
RER
V
M
0.2µm
4.15

Fig. 4.16: The fourth-stage cuticle (C4) of Nematospiroides dubius as it begins to differentiate beneath the third-stage cuticle (C3). The oblique section shows the C4 is composed of a series of parallel electron dense lines, which if viewed from the top of the micrograph (→), appear to be made up of rows of "honeycomb" subunits (HC). Mitochondria (M) and ribosomes (R) are present in the epidermis and the cisternal space (CS) of the endoplasmic reticulum appears enlarged. A "tram-line" pattern is present within the C4.

Fig. 4.17: Glimpses of the trilaminate epicuticle (circled) are apparent within the outer dense region of the fourth-stage cuticle of Nematospiroides dubius (C4), beneath the third-stage cuticle (C3). The epidermal region contains mitochondria (M) and Golgi complexes (G). Endoplasmic reticulum, with an enlarged cisternal space (CS) is extensive. Note the "myelin figure" (MF) in the left of the micrograph.


C3
HC
C4
HC
R
CS
CS
R
M
0.2μm
4.16

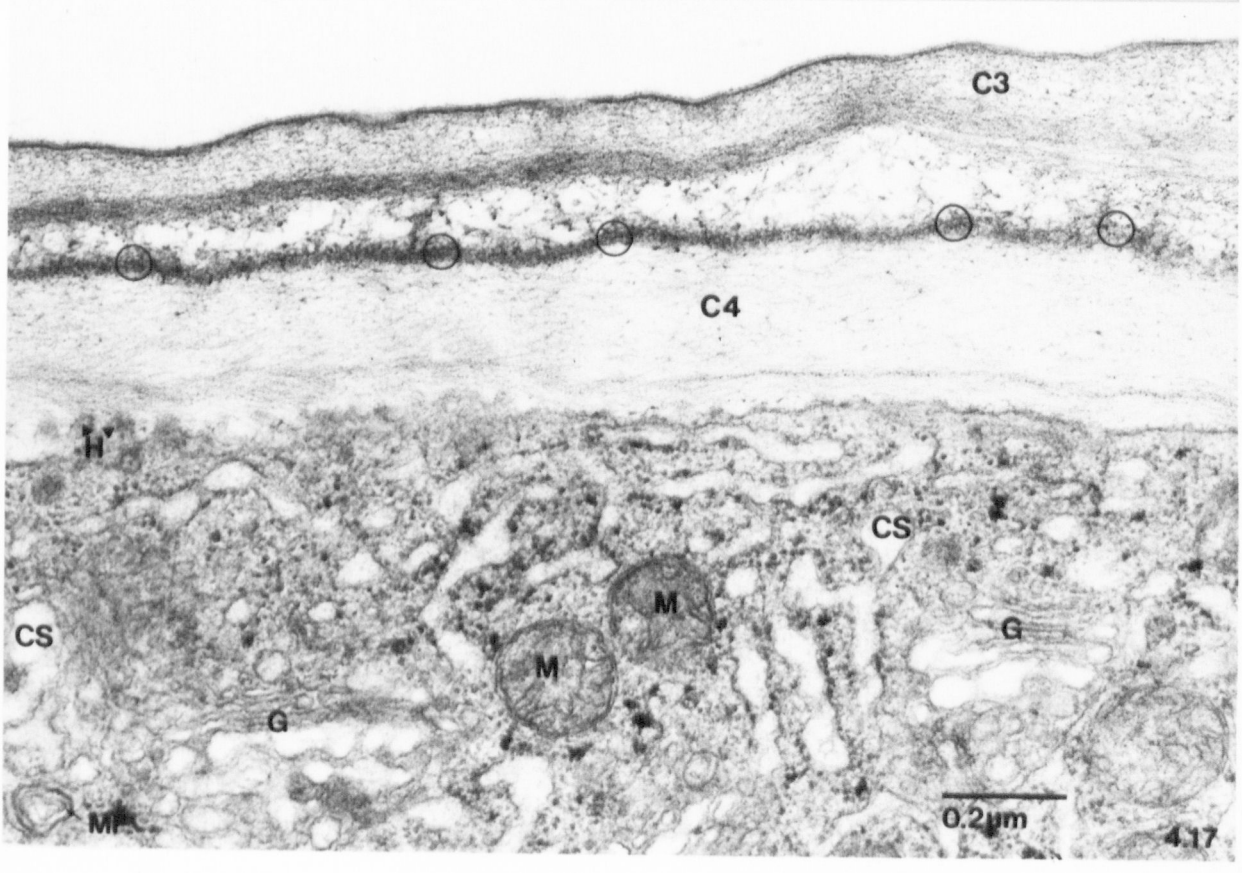
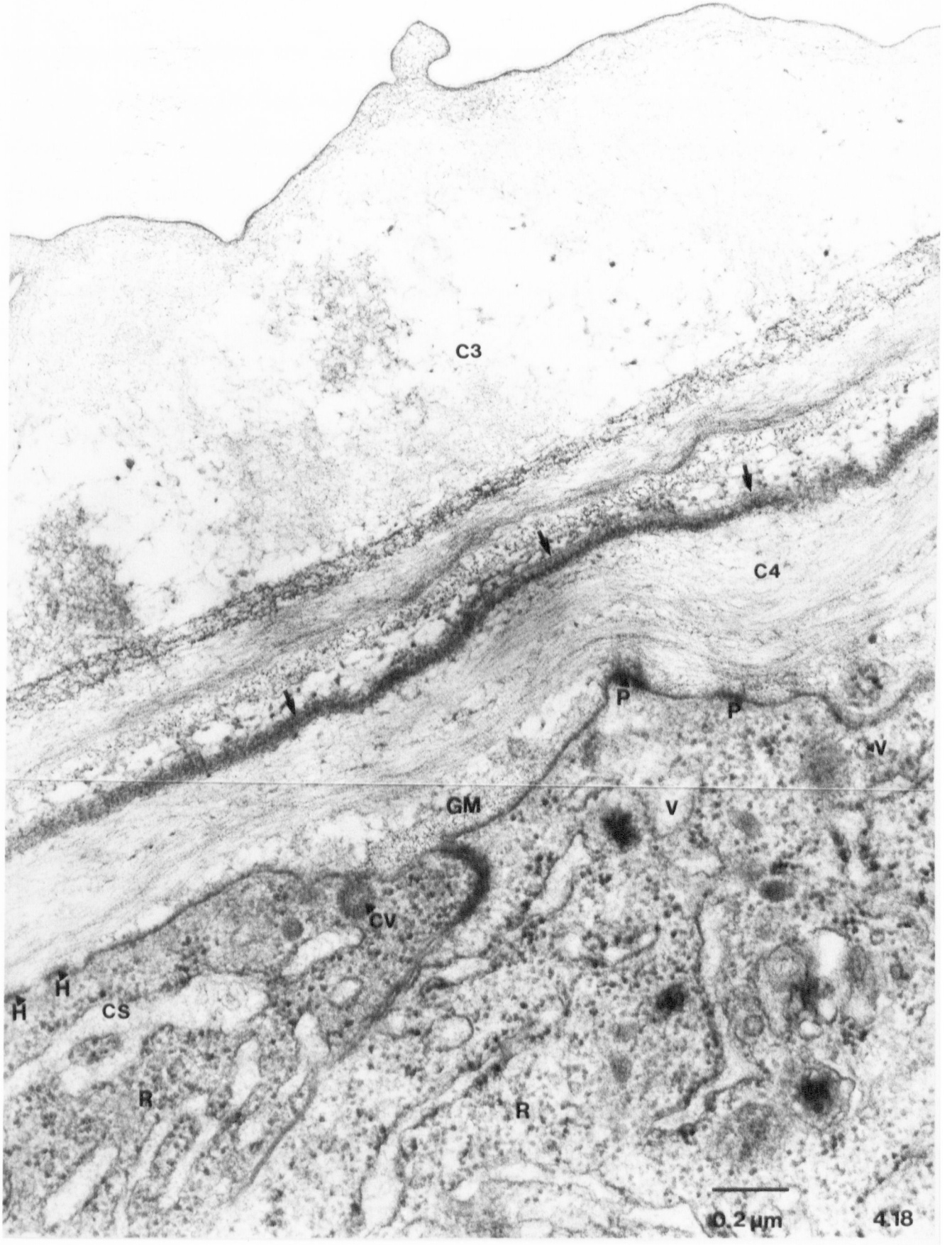

C3
C4
H
CS
M
M
CS
G
G
MF
0.2μm
4.17

Fig. 4.18: Several distinct layers are visible in the fourth-stage cuticle of Nematospiroides dubius (C4) as it develops beneath the third-stage cuticle (C3). The epicuticle is distinct (→). Granular material (GM) is present near the dense patches (P) that occur along the epidermal membrane. Also present within the lateral region are ribosomes (R), vesicles enclosing an enlarged cisternal space (CS) and what appear to be coated vesicles (CV). Hemidesmosomes (H) occur between epidermis and C4.


C3
C4
P
P
V
GM
V
CV
H
H
CS
R
R
0.2 µm
4.18

hemidesmosomes between the new cuticle and the epidermis (Fig. 4.18).

As depicted in Figs 4.19 and 4.20, the new fourth-stage cuticle underwent further differentiation before the third-stage cuticle was discarded. Within the cortical zone of the M-shaped "plicae", for example, were dense patches, below which at least two fibrous layers could be detected. The epicuticle was clearly present on the surface of the new cuticle as a trilaminate structure and, below the cuticle, the epidermal surface appeared convolted (Fig. 4.19)..

Early events in the formation of the adult cuticle were not followed closely but some of the processes by which material was added to the developing cuticle were noted. As in the formation of the fourth-stage cuticle, the epidermis appeared active even when cuticle formation was well advanced (Figs 4.21 to 4.24). The epidermis contained numerous vesicles, some of which appeared to fuse with the epidermal membrane (Figs 4.22 to 4.24). Structures resembling coated vesicles and multivesicular bodies were present (Fig. 4.21), and membranous "whorls" or "myelin figures" occurred between cuticle and epidermis.

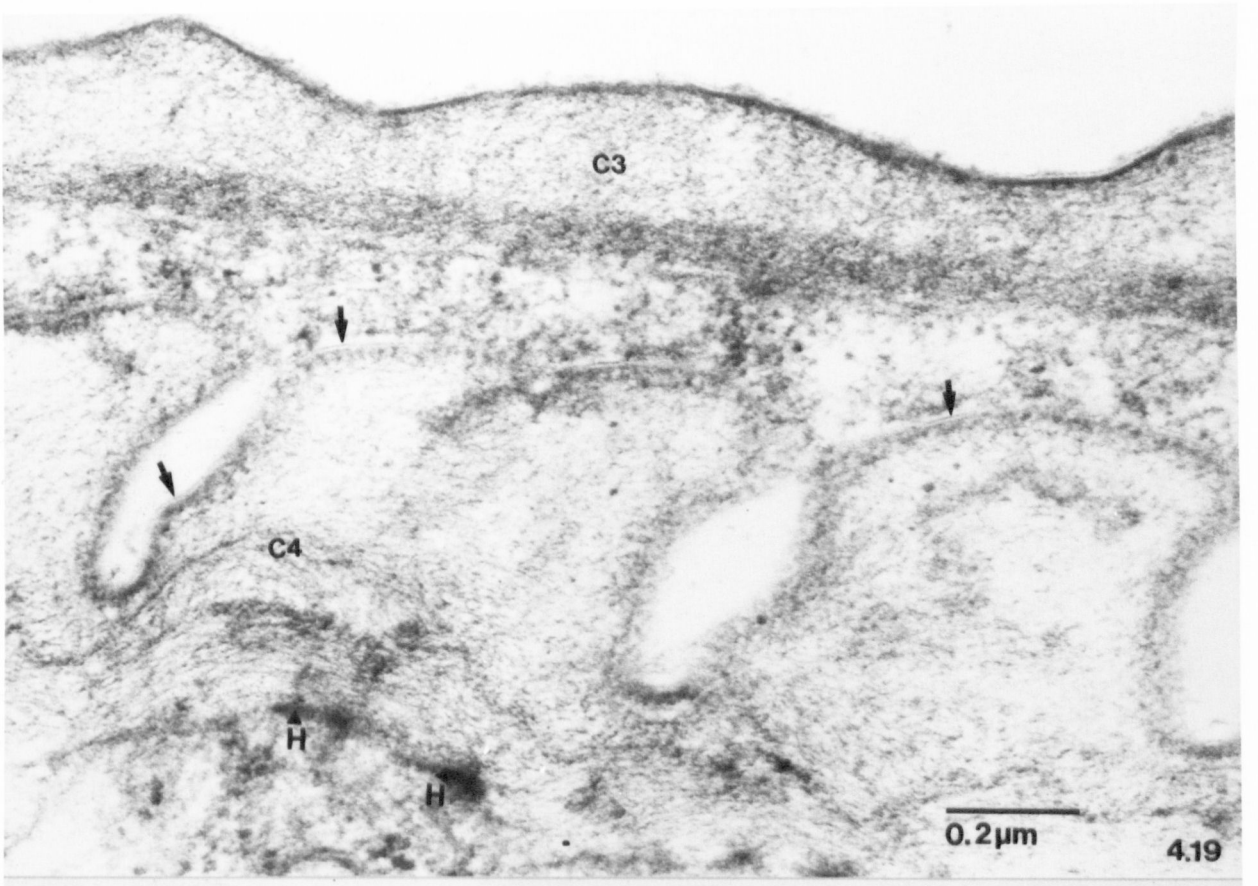
4.4 DISCUSSION

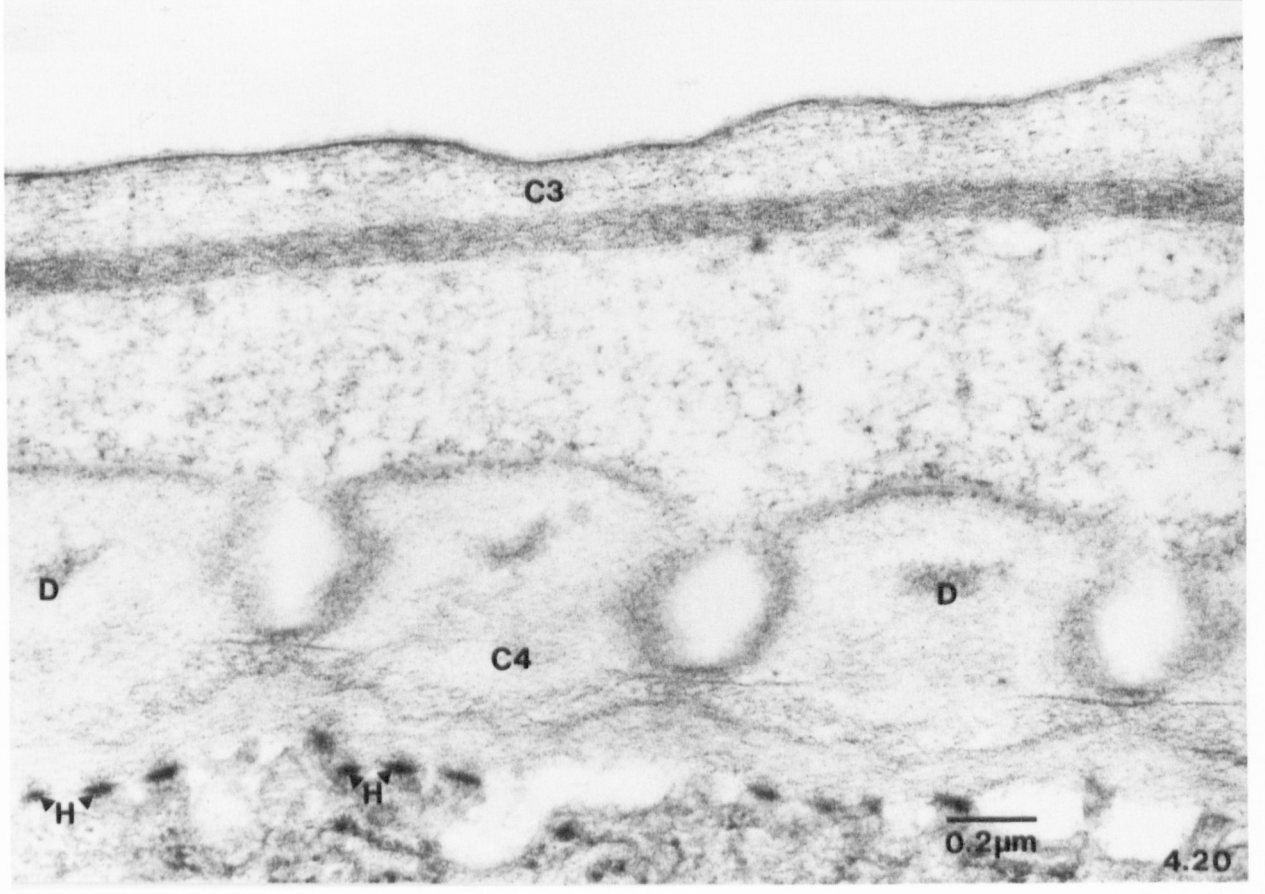
The results of this ultrastructural study of the formation of the fourth-stage cuticle of *N. dubius* indicate that the epicuticle is an extracellular structure.

4.4.1 Formation of the epicuticle

The sequence of micrographs presented here satisfy the criteria that were proposed as being necessary to adequately describe the

Figs 4.19 and 4.20: Micrographs depicting events prior to the loss of the third-stage cuticle (C3) of Nematospiroides dubius. The fourth-stage cuticle (C4) is substantial, with the epicuticle clearly present along its surface (→). The C4, which is attached to the convoluted epidermal surface via hemidesmosomes (H), is composed of several layers. Note the dense areas (D) within the C4.


C3
C4
H
H
0.2µm
4.19


C3
D
D
C4
H
H
0.2µm
4.20

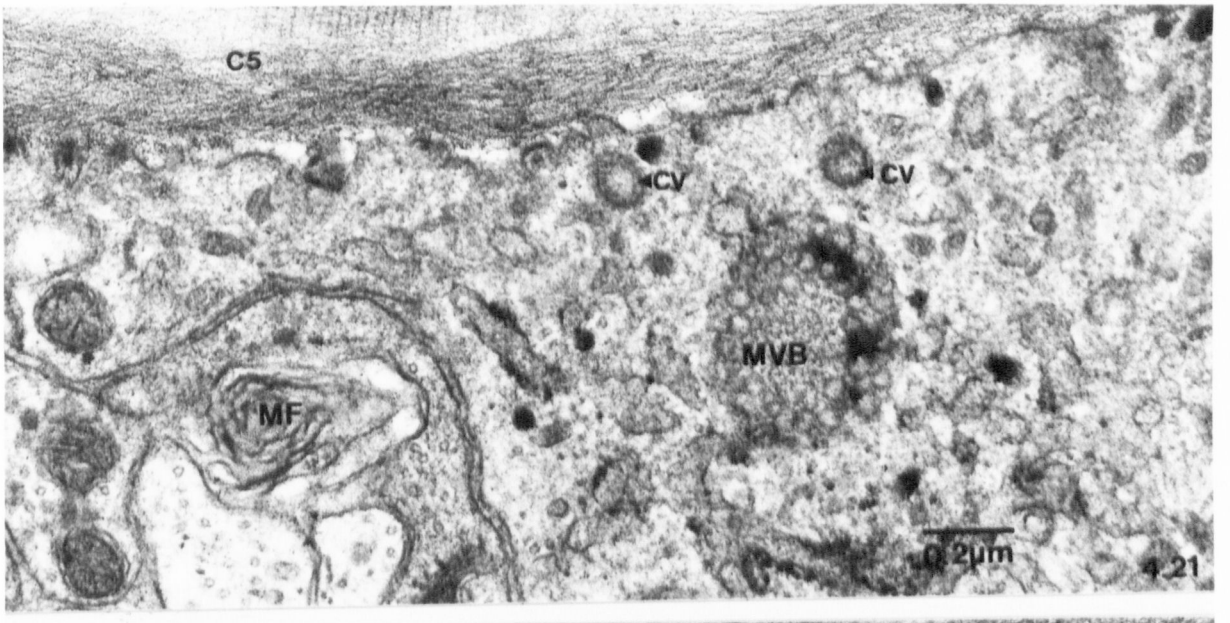
Figs 4.21 to 4.24: Micrographs of late fourth-stage larvae of Nematospiroides dubius undergoing the final moult. Formation of the adult cuticle (C5) is well advanced with the basal fibre layers clearly present.

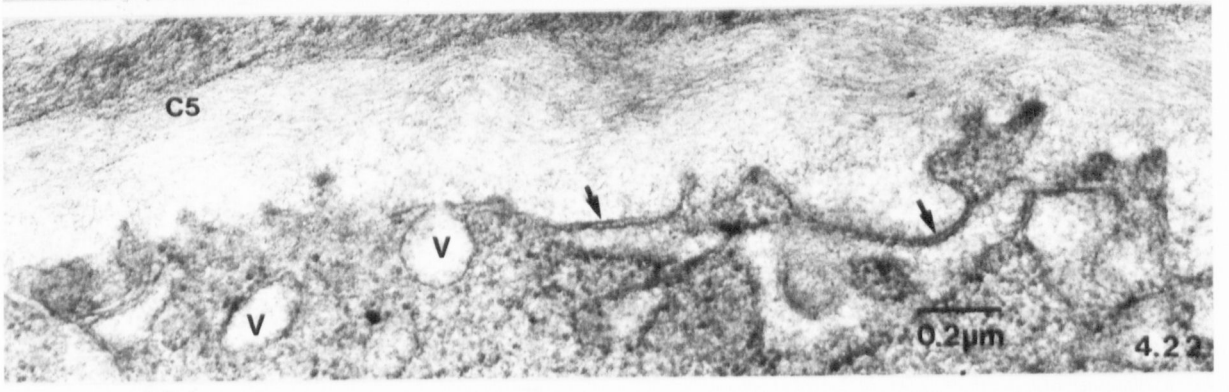
Fig. 4.21: A multivesicular body (MVB) and several coated vesicles (CV) can be seen within the epidermal region. Note the myelin-like figure (MF).

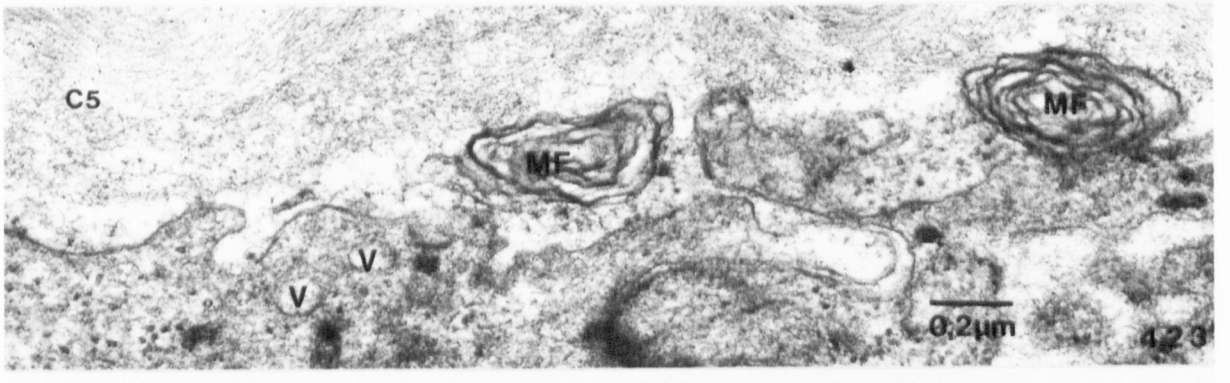
Fig. 4.22: The trilaminate structure of the epidermal membrane (→) is distinct. One vesicle (V) has formed an omega figure as it fuses with the epidermal membrane.

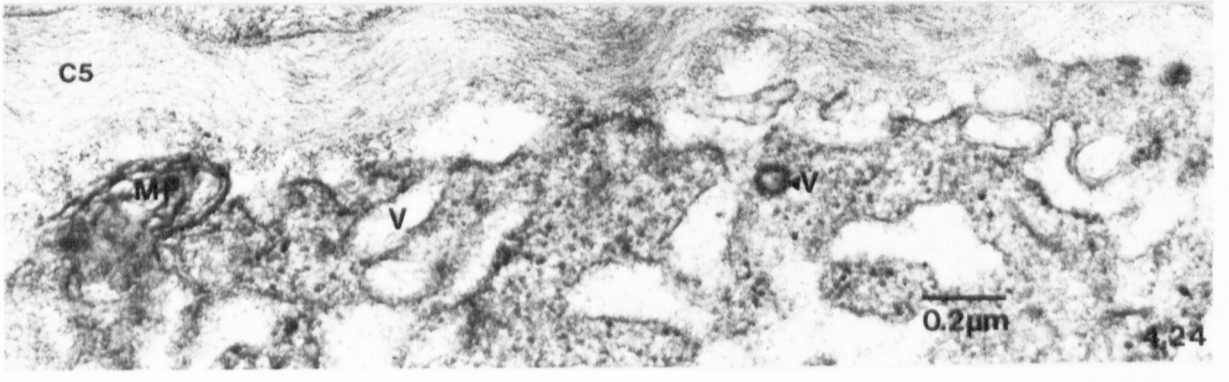
Fig. 4.23: Myelin-like figures (MF) lie between the new C5 and epidermal region which contains many vesicles (V).

Fig. 4.24: Late during the cuticle formation of the C5, the epidermal region contains many vesicles (V). Note the myelin-like figure (MF) between the C5 and epidermal membrane.


C5
CV
CV
MVB
MF
0.2µm
4.21


C5
V
V
0.2µm
4.22


C5
MF
MF
V
V
0.2µm
4.23


C5
MF
V
V
0.2µm
4.24

formation of the epicuticle (see Section 4.1). Firstly, the intact epidermal membrane was seen attached to the third-stage cuticle (Figs 4.2 to 4.9). Secondly, micrographs depicting the epidermal membrane with undifferentiated filamentous material between it and the third-stage cuticle were presented (Figs 4.10 to 4.13). Finally, the epidermal membrane with the overlying filamentous material differentiating into layers was illustrated (Figs 4.14 to 4.20).

Thus, during the formation of the fourth-stage cuticle of N. dubius, precursor elements were deposited on the surface of the epidermis. These elements subsequently differentiated to produce a triple-layered structure, the epicuticle. The remaining components of the new cuticle were then secreted by the epidermis, beneath the differentiating epicuticle. The fourth-stage cuticle of N. dubius was clearly revealed as an extracellular structure secreted by the epidermis.

As already indicated, caution is required when arranging micrographs from several worms so that they together describe an event. There is reason to suppose, however, that the micrographs presented here cannot be arranged in any other logical sequence.

Firstly, the epicuticle was not discernible as a trilaminate structure until the new cuticle measured about 150 nm (Figs 4.14 to 4.18). Thus, until the new cuticle reached a substantial size, only one triple-layered structure, the epidermal membrane, was present.

Similarly, Bonner and Weinstein (1972a) observed that differentiation of the epicuticle of the fourth-stage cuticle of N. brasiliensis only took place when the new cuticle measured between 100 and 250 nm in thickness, while the adult cuticle of N. dubius was at least 60 to 80 nm thick before the epicuticle appeared trilaminate (measurements were taken from Fig. 8 and Fig. 13, respectively in Bonner & Weinstein, 1972a). Martinez-Palomo (1978) also noted that the

earliest sign of layering within the fibrillar elements making up the first-stage cuticle of O. volvulus took place when the new cuticle was approximately 65 nm thick. The outer region of the new cuticle then became progressively more organized until it measured 140 nm in thickness.

A number of a different studies, therefore, have concluded that the epicuticle differentiates only when a substantial portion of the precursor elements making up the new cuticle are present. As Bonner and Weinstein (1972a) pointed out, it is the early events in cuticle formation that are all important in deciding where the epicuticle forms but in some studies, for example Lee (1970), Bonner et al. (1970) and Bird (1977), these were not recorded. In fact, where the very early stages of cuticle formation have been observed (Bonner & Weinstein, 1972a; Martinez-Palomo, 1978), there is no question about which trilaminate structure was present earliest.

A second line of evidence in support of the notion that the epidermal membrane was always present as the cuticle formed above it, comes from noting the structure of the epidermal membrane throughout the process. Although the trilaminate structure of the epidermal membrane was not always visible (Figs. 4.2 and 4.3), following apolysis the epidermal membrane could be identified by the presence of electron dense regions or patches that extended on either side of it (Figs 4.12 and 4.18). It is not clear whether these patches represent sites where hemidesmosomes were present prior to apolysis, but they were apparent from the very early stages of cuticle formation (compare Fig. 4.3 with Figs 4.6 to 4.10).

Similar electron dense patches were present along the epidermal membrane of N. brasiliensis throughout the formation of the fourth-stage cuticle (Bonner & Weinstein, 1972a), as well as along the epidermal

membrane of the developing first-stage larva of O. volvulus (Martinez-Palomo, 1978). Some micrographs presented in the latter study were not exceptionally clear but the epidermal membrane could always be identified by the occurrence of dense patches below the filamentous coat that underwent progressive layering.

To summarize, the presence of the epidermal membrane throughout cuticle formation, together with the observation that the epicuticle only differentiated when the new cuticle measured about 100 nm in thickness, leaves little doubt as to the extracellular nature of the epicuticle. At no time during the formation of the fourth-stage cuticle of N. dubius was the new epicuticle continuous with the plasma membrane of the epidermis, so clearly the epidermal membrane does not modify to form the epicuticle (Bird, 1980). Instead this demonstration that the cuticle is an extracellular structure confers credibility on Locke's (1982) hypothesis that the nematode epicuticle should be thought of as an envelope. The early stages of cuticle formation have been studied closely only in a few species of nematode but it seems likely that the way in which a new cuticle is formed would be similar in all nematodes.

4.4.2 Synthesis of the cuticle

The synthesis of the epicuticle must be considered in conjunction with the formation of the inner three zones of the cuticle. Little is known about the mechanisms of cuticle formation in nematodes; much of the early work focused on the order in which the layers of the cuticle appeared, rather than on the way precursor elements were organized to form the new cuticle. Of particular interest is how the precursor elements reach the surface of the epidermis and the way in which these elements are then organized to form the cuticle. Of course, the problem

of maintaining adhesion between the active epidermal membrane and the cuticle must be kept in mind.

The bulk of the precursor elements of the new fourth-stage cuticle of N. dubius appeared to be secreted on to the surface of the epidermis via a variety of vesicles. Whether the earliest precursors were also deposited on the outside of the epidermis by way of vesicles or whether they moved through the plasma membrane is unknown because the filamentous precursors measured about 75 nm when they were first observed in this study. Certainly the initial deposition on the surface of the epidermal membrane was added to by vesicles that fused with the epidermal membrane (for example, Figs 4.10 to 4.13, 4.15, 4.18 and 4.22).

The contents of some vesicles appeared similar to the filamentous material between the epidermis and the old cuticle; an observation consistent with the idea that the contents of the vesicles is deposited on the surface of the epidermis (see for example, Figs 4.11, 4.15 and 4.18). A similar observation was made by Bonner and Weinstein (1972a) and Lee (1970).

In contrast, during cuticle formation in insects, although precursor elements were observed to aggregate spontaneously above the plasma membrane, no vesicles were observed to fuse with the membrane (Locke, 1966). Instead precursor elements must have passed through the plasma membrane. Similarly, when gram-negative bacteria produce or repair the outer membrane or envelope, precursors synthesized in the cell are apparently pushed through the plasma membrane and inserted into the outer membrane (Bayer, 1979; Ghuysen, 1977; Hammond, Lambert & Rycroft, 1984).

A number of different types of vesicle were seen in the epidermis of N. dubius during cuticle formation. Vesicles resembling coated vesicles, for example, were located close to the apical surface of the

epidermis (Figs 4.15, 4.18 and 4.21). Bonner and Weinstein (1972a; 1972b) also found coated vesicles within the epidermis of N. brasiliensis during its fourth moult. Coated vesicles are involved in intracellular transport between organelles, endocytosis and membrane recycling and have been implicated in the two phase transport of molecules from the endoplasmic reticulum to the Golgi complexes and then from the Golgi complexes to the plasma membrane (Alberts, Bray, Lewis, Raff, Roberts & Watson, 1983; Rothman & Fine, 1980). It seems likely, therefore, that the coated vesicles observed in the epidermis of N. dubius during cuticle formation were transporting precursor molecules to the surface of the epidermis.

Also present within the epidermis of larvae of N. dubius during cuticle formation were multivesicular bodies (Figs 4.5, 4.11, 4.14 and 4.21). Although multivesicular bodies have been located in a number of species of nematode, their function remains unknown (Bonner et al., 1970, Bonner & Weinstein, 1972b, McClaren, 1972, Webster & Gordon, 1974). In vertebrate cells, multivesicular bodies are usually, but not always, present in cells involved in endocytotic activity (Fawcett, 1981; Pasteel, 1973). Endocytosis, however, seems an unlikely role for organelles in a cell actively secreting cuticular material.

As well as coated vesicles and multivesicular bodies being present within the epidermis of N. dubius as a new cuticle is formed, the lumen of the endoplasmic reticulum was sometimes enlarged giving the appearance of membrane-bound cavities. Such structures were often located close to the surface of the epidermis and, in some cases, appeared to be fused with the epidermal membrane so exposing their contents on the surface of the epidermis (Figs 4.12, 4.13, 4.17 and 4.22 to 4.24).

Myelin-like figures or lamellar bodies were frequently observed

during cuticle formation. Although usually present between cuticle and epidermis, they could sometimes be seen within the epidermis (Figs 4.17, 4.21, 4.23, 4.24). Similar structures have been observed between the cuticle and epidermis in third-stage larvae of N. brasiliensis prior to infection (Bonner & Weinstein, 1972a), and within the tissue of immune-damaged S. ratti that had been transplanted in a new host (Moqbel, McClaren & Wakelin, 1980).

The function of these myelin-like figures is unknown but they are thought to be composed of whorls of hydrated phospholipid in which the spacing between concentric layers is determined by the type of phospholipid involved (Henn & Thompson, 1969; Papahadjopoulous & Miller, 1967). Moqbel et al. (1980) suggested the myelin-like figures represented an overproduction of membrane in S. ratti. Alternatively the figures may be a modification of, or an intermediate in, the biogenesis of a membrane system, such as the endoplasmic reticulum (Mollenhauer, Morre & Jelsema, 1978). On the other hand, myelin-like figures have also been associated with the degeneration of tissues (Fox, 1970; Reader, 1973). Although the function of the myelin-like figures in N. dubius is not understood, it seems likely that their presence is not an artefact but an indication of surplus membranous components.

Thus, this ultrastructural study of cuticle formation in N. dubius, together with studies on other nematodes, indicates that vesicles deposit the bulk of the cuticle on to the surface of the epidermis. The likelihood that these elements then form the structured cuticle by self-assembly will now be considered.

4.4.3 Role of self-assembly in cuticle formation

Ultrastructural evidence from the fourth moult of N. dubius

suggests that the epicuticle and inner three zones of the new cuticle form, in part at least, by self-assembly. The final dimensions of the fourth-stage epicuticle of N. dubius, for example, were the same as the initial structure. As soon as the fourth-stage epicuticle could be definitely identified, it measured between 11 and 13 nm in thickness which was approximately the same as the epicuticle of a late fourth-stage larva (compare Fig. 4.18 with Fig. 3.12). According to Locke (1982), this suggests that the epicuticle, in common with other envelopes, assembles directly from precursors and is not stretched and squeezed to achieve the final dimensions.

Similarly, the epicuticle of the few species of nematodes that have been studied in detail also appears to form from precursor elements. Undifferentiated precursors were observed on the epidermal surface where they underwent progressive layering (Bonner & Weinstein, 1972a; Martinez-Palomo, 1978).

The formation of the envelope or peristracum of the molluscan shell, on the other hand is somewhat different (Saleuddin, 1975). In Helistoma, the peristracum is secreted by underlying cells in a series of trilaminate units, about 100 to 200 nm long and 10 to 13 nm wide. These units are released on to the surface via an exocytotic process before becoming aligned to form a single layer covering the shell. In this mollusc, therefore, the envelope assembles from a series of relatively large individual units.

Just as the epicuticle of N. dubius appears to assemble from precursor elements, so too does the remainder of the cuticle. When an oblique section of an early stage of cuticle formation is viewed, the cuticle is composed of rows of honeycomb subunits (Fig. 4.16). When the same micrograph is viewed from a different angle, the cuticle appears to be made up of a series of parallel, electron dense lines or plates

reminiscent of the pattern seen within the spines of the adult cuticle (see Fig. 3.15). It is of interest to note that hexagonal subunits can also be discerned in oblique sections of the adult cuticle (see Fig. 3.15).

There are theoretical reasons to suppose that self-assembly of precursor elements outside the epidermis plays a role in cuticle formation in nematodes. In the first place, the formation of large fibrous proteins within a cell would obviously be limited by the size of both the protein and the cell.

In addition, isolated protein molecules can self-assemble to form aggregates, the structure of which is determined by their physical properties. One example of self-assembly in action occurs in the tobacco-mosaic virus (Fraenkel-Conrat & Williams, 1955). If the dissociated ribonucleic acid and protein subunits are mixed together in solution, they can recombine to form active virus particles. Similarly, isolated subunits of the crystalline surface layer of bacteria self-assemble into lattices which are identical to those found surrounding intact cells (Sletyr & Messner, 1983).

Two or more of the same type of protein molecule tend to form aggregates of noncovalently assembled subunits (Cantor & Schimmel, 1980; Walton, 1981). For example, where the binding site of a protein is complementary to a region on its surface that does not include the binding site itself, the chain of subunits will be arranged into a helix, as in the actin filament (Walton, 1981). Keeping in mind the ordered aggregation of subunits making up the new cuticle in Fig. 4.16, it is interesting to note that protein subunits can assemble into flat sheets in which subunits are arranged in hexagonal arrays (Alberts et al., 1983).

The formation of collagen fibrils is also largely due to the

tendency of collagen molecules to self-assemble, but in this case the cell surface is thought to influence the site and rate of fibril assembly (Hay, 1981). Collagen polypeptides that are synthesized on membrane-bound ribosomes spontaneously form triple-stranded, helical procollagen molecules within the lumen of the endoplasmic reticulum. Associated with these procollagen chains are extension peptides which prevent the procollagen chains from assembling into collagen molecules. Once outside the cell, however, procollagen peptidases remove the extension peptides and the collagen molecules assemble into fibrils.

There are, therefore, theoretical grounds to suppose that the precursor elements of the cuticle of N. dubius become ordered by self-assembly. Furthermore, it seems likely that the epidermis is involved in regulating self-assembly of the extracellular molecules.

One possible candidate for the role of regulating cuticle assembly may be the dense patches present along the epidermal membrane of N. dubius during cuticle formation. These patches are reminiscent of so called "plaques" found on the apical membrane of epidermal cells of insects which are thought to be involved in attaching cuticle and epidermis (Locke & Huie, 1979). Because the plaques are present near sites where the fibrous cuticle and cuticulin first appear during cuticle formation, they are presumed to have a role in the synthesis and/or deposition and orientation of the cuticle precursor elements (Locke & Huie, 1979).

The dense patches present along the epidermal membrane of nematodes may have a function similar to that of plaques in insects. In N. dubius, the dense regions extend on either side of the epidermal membrane and appear to be intimately associated with the new layers of precursor elements. Figure 4.18, for example, demonstrates that the new

cuticle has an extra layer, a granular deposit, in the vicinity of such dense patches compared with the surrounding cuticle.

Moreover, like the plaques found in insects, the patches may represent potential sites of attachment between epidermis and cuticle. During the early stages of cuticle formation the hemidesmosomes, which are believed important in attaching the cuticle to the epidermis (Figs 4.6 and 4.15, Lee, 1970; Sommerville, 1982), either diminish in size or even disappear when the cuticle and epidermis separate (compare Figs 4.3 to 4.6, 4.10, 4.11 and 4.13). It is tempting to speculate that the dense patches which appear early in cuticle formation, not only have a role in the deposition and/or assembly of precursor molecules, but are also potential sites of hemidesmosomes.

Thus, during the formation of the fourth-stage cuticle in N. dubius, the precursors of the new cuticle were deposited on the surface of the epidermis, largely by vesicles. It seems likely that the precusor elements were then structured into the new cuticle, partly by self-assembly and partly under the influence of the epidermal membrane.

CHAPTER 5

A FREEZE-FRACTURE STUDY OF THE EPICUTICLE OF N. dubius

5.1 INTRODUCTION

Using freeze-fracture replication it is possible to visualize the interior of a biological membrane (Branton, 1966; Pinto da Silva & Branton, 1970; Tillack & Marchesi, 1970), or to examine the fine details of extracellular structures (Altner, 1975; Filshie & Smith, 1980; Flower & Walker, 1979; Greven & Robenek, 1983; Lee & Nicholls, 1983; Noirot, Noirot-Timothée, Smith & Cayer, 1978). It is also possible to infer some chemical properties of a membrane from the way that it freeze-fractures (Verkleij and Ververgaert, 1978).

Freeze-fracture involves cleaving biological tissue at a low temperature (< -100°C) in a vacuum (< 1 x 10^{-5}mm Hg). A replica of the exposed tissue is produced by shadowing it with a heavy metal, such as platinum, and covering it with a backing of evaporated carbon before dissolving away the tissue (Branton, 1966; McNutt, 1977; Zingsheim & Plattner, 1976).

When biological membranes are fractured at low temperature, they split through the hydrophobic mid-plane revealing two possible fracture faces (Daemer & Branton, 1967; McNutt, 1977; Pinto da Silva & Branton, 1970; Tillack & Marchesi, 1970; Verkleij & Ververgaert, 1978). One face is the hydrophobic side of the outer leaflet of the membrane, referred to as the E fracture (F) face , while the second is the hydrophobic side of the inner or protoplasmic half-membrane, referred to as the P fracture (F) face (Branton, Bullivant, Gilula, Karnovsky, Moor, Mühlethaler, Northcote, Packer,

Satir, Satir, Speth, Staehlin, Steere & Weinstein, 1975). In addition, the fracture plane can pass along the upper or lower surface of the membrane producing two true surfaces, the external (ES) and the protoplasmic (PS) surface (Branton et al., 1975).

The fracture faces are studded with particles, called intramembrane particles (IMP's) which vary in density and arrangement among different membrane systems (Branton, 1966, Goodenough & Stoeckenius, 1972; McNutt, 1977; Zingsheim & Plattner, 1976). Metabolically active membranes, for example, tend to exhibit more particles than those that are less active (Branton, 1971; Humbert, Pricam, Perrelet & Orci, 1975; Pinto da Silva & Miller, 1975). In general, the PF face contains numerous particles which measure about 10 nm in diameter, while the EF face has fewer particles (Zingsheim & Plattner, 1976). Depressions or pits are sometimes visible on the fracture faces (Zingsheim & Plattner, 1976; Verkleij & Ververgaert, 1978).

The IMP's seen on the faces of fractured biological membranes are usually protein or aggregates of protein molecules (McNutt, 1977; Zingsheim & Plattner, 1976). Evidence for this comes from a number of different sources. Recombination studies using artificial lipid bilayers and purified protein, as well as studies using membranes known to lack a specific protein, have proved particularly useful (Montal, 1974; Ruben & Telford, 1980; Simpson, 1979; Yu & Branton, 1976). More recently, Aguas & Pinto da Silva (1983) used fracture-label combined with surface labelling techniques to neatly demonstrate that sites on the PF face of membranes, which bound to wheat germ agglutinnin (WGA), corresponded to transmembrane WGA-binding glycoproteins intercalated across the membrane.

Membranes containing a high proportion of lipopolysaccharides can be distinguished from plasma membranes by their freeze-fracturing characteristics (Verkleij & Ververgaert, 1978). A substantial portion of the outer membrane of Gram-negative bacteria, for example, is made up of lipopolysaccharides (Bayer, 1981; Beveridge, 1981). Unlike a plasma membrane, the bacterial envelope is difficult to freeze-fracture, and once fractured, the PF face appears highly pitted (Bayer, Koplow & Goldfine, 1975; Smit, Kamio & Nikaido, 1975; Van Alphen, Verkleij, Leunissen-Bijvelt & Lugtenberg, 1978; Van Gool & Nanninga, 1971; Verkleij, Van Alphen, Bijvelt & Lugtenberg, 1977).

Using freeze-fracture replication, it appears that the epicuticle of nematodes varies in compostion. The outer two layers of the cuticle of first-stage larvae of T. spiralis did not split when freeze-fractured, but the epicuticle of other species of nematode has been found to cleave at low temperature (Bird, 1984; Lee & Bonner, 1982; Lee et al., 1984).

Where the epicuticle of nematodes has fractured, differences in the particles present on the fracture faces have been recorded. Bird (1984) located densely packed particles on the PF face of second-stage cuticle of M. javanica, while Lee and Bonner (1982) reported minute particles on a fractured face of the epicuticle of N. brasiliensis.

In light of the conflicting findings relating to the epicuticle of nematodes, it was of interest to establish firstly, if the epicuticle of N. dubius would split when freeze-fractured and secondly, if particles are present on the fracture faces.

5.2 MATERIALS AND METHODS

Third-stage larvae and adult worms were freeze-fractured according to the following schedule.

Adult worms, males and females combined, which were collected as described earlier (see Section 2.2.2), were washed in 0.1M Sörensen's phosphate buffer at pH 7.2 and briefly fixed in 3% glutaraldehyde in the same buffer. The worms were then cut into small pieces and fixed for a further 90 minutes before being rinsed in 3 changes of 0.1M Sörensen's phosphate buffer over 30 minutes. The third-stage larvae were fixed in the same manner except they were not cut because large numbers of worms needed to be used.

The tissue was then infiltrated with glycerol in water up to a concentration of 30% over 2 days. Where necessary, worms were stored in 30% glycerol at 4°C. The tissue was placed in the central well of the specimen holder and the excess water was removed using filter paper. The tissue was then frozen in liquid Freon 22, cooled by liquid nitrogen, followed by liquid nitrogen alone.

Worms were fractured at -110°C in a Balzers BAF 301 Freeze-etching apparatus. The fractured surface was not etched but shadowed immediately with a 2 nm thickness of platinum and a backing of carbon (15 to 20 seconds) at a pressure of 3×10^{-6}mm Hg and an angle of 45°.

Replicas were cleaned overnight in sodium hypochlorite, followed by several hours in 20% chromic acid and four rinses in distilled water.

In all, 7 replicas of adult worms were made and 5 of third-stage larvae. Only 1 of these 12 replicas showed substantial fracturing of the epicuticle. For both stages of N. dubius, tissue was also fractured unfixed in an effort to promote fracturing, but ice damage made it difficult to locate the tissue. The small bits of tissue that could be

discerned, however, showed no increase in tendency to fracture.

Replicas were mounted on 100 mesh grids and observed and photographed in a Jeol 100S electron microscope. To obtain a measure of particle size and density, electron micrographs were taken at a magnification of 50 000 times and enlarged 6 times. The regions of fracture face suitable for quantifying particle density and size were limited. For PF faces, an area of 1 μm^2 was analyzed but only 0.5 μm^2 of the EF faces was suitable. Particles were counted by dividing the whole region into 0.01 μm^2 squares and because it was difficult to recognize individual particles, two counts were made over each area. To obtain an idea of particle size, all particles in an area of 0.25 μm^2 were measured to the nearest 0.1 mm using a Polaron measuring eyepiece. Micrographs were positioned with the shadow coming from the bottom of the page, where possible.

5.3 RESULTS

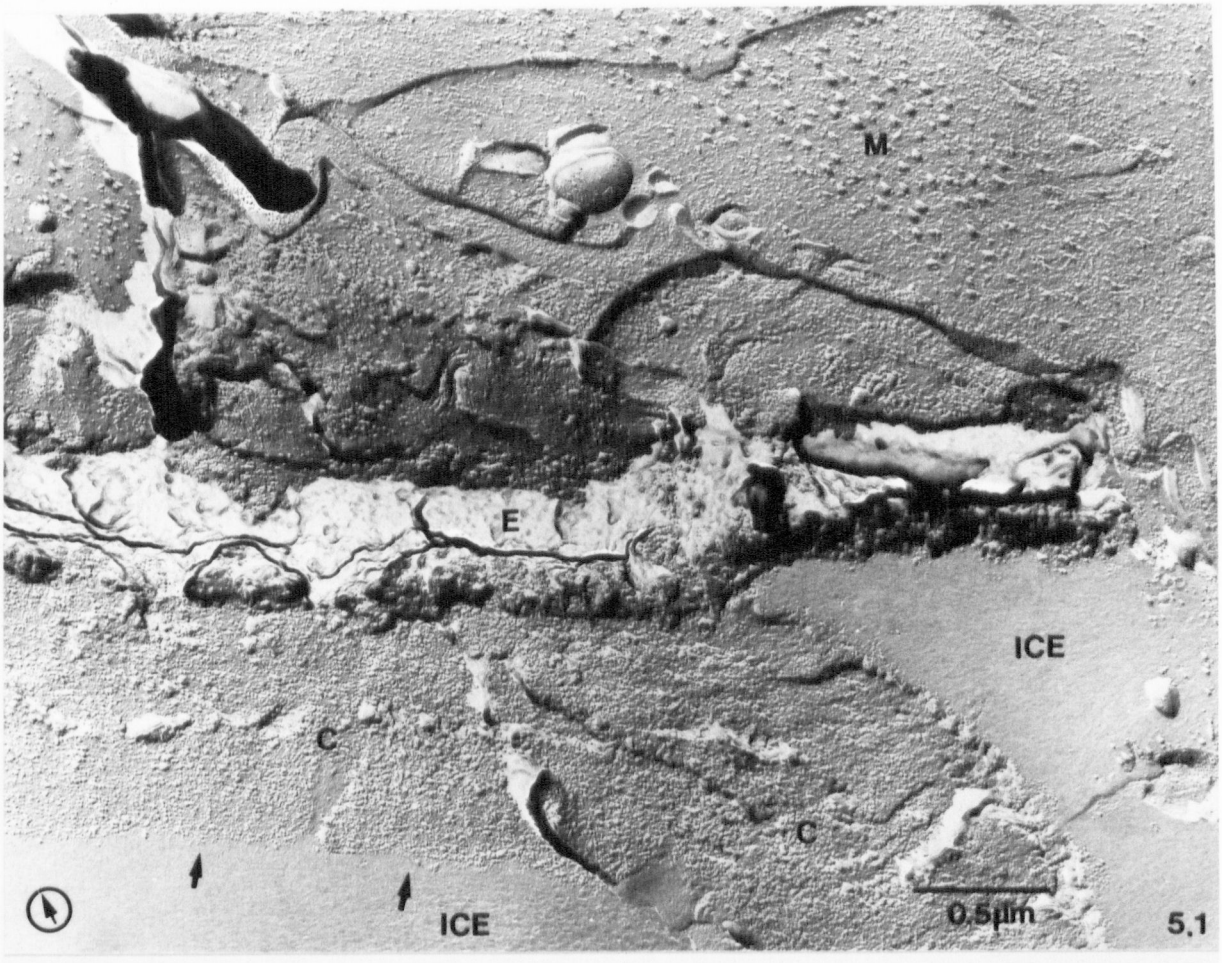
Nematode tissue was easily identified when replicas were viewed in an electron microscope (Figs 5.1 and 5.2). Muscle fibres, which exhibited a characteristic pattern, were initially used as reference points in locating the cuticle but the homogeneous, granular appearance of the cuticle was quickly recognized (Fig. 5.1). Replicas were scanned at low magnification until cuticular tissue was located. The outer edge of the cuticle, that is the side away from the muscle or epidermis, was then scanned at high magnification for signs of a fractured surface (Fig. 5.2).

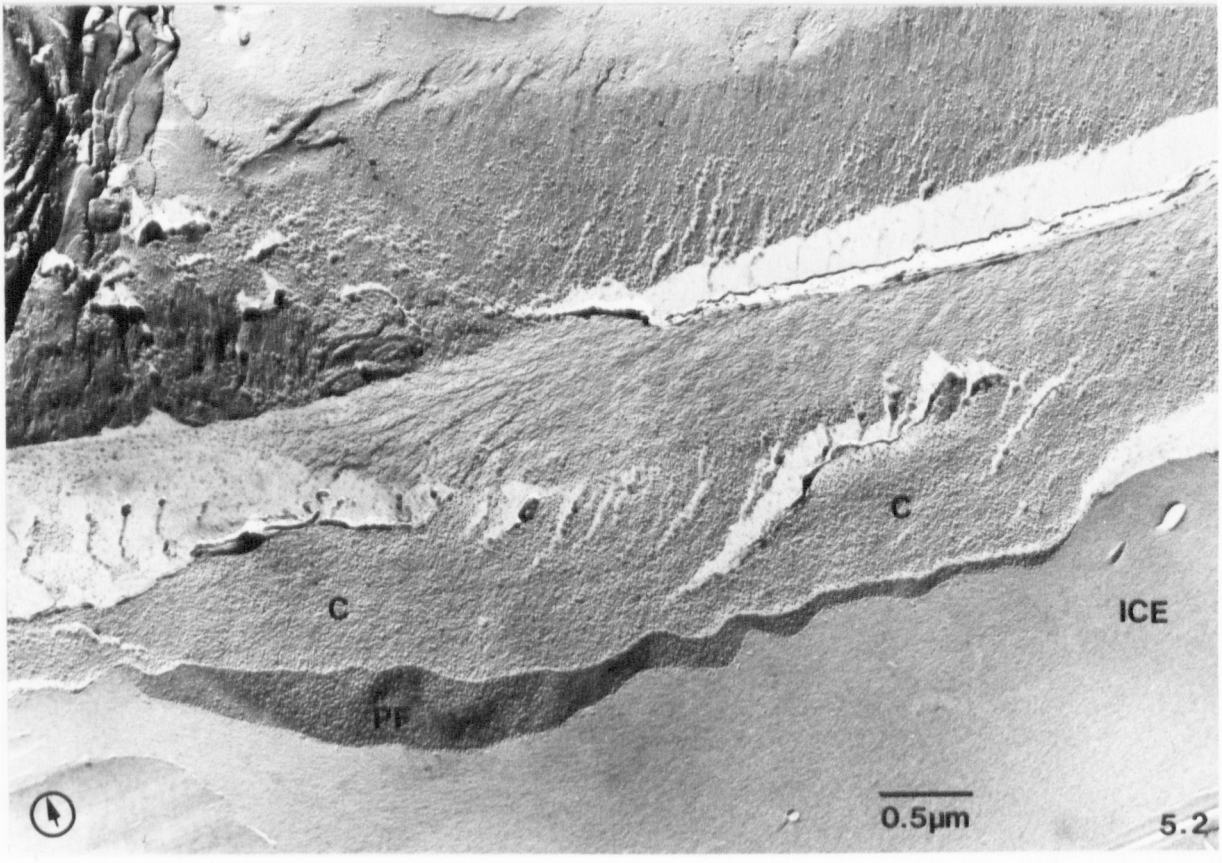
The epicuticle of adult N. dubius split when freeze-fractured to expose a PF face (Figs 5.3 to 5.9) and an EF face (Figs 5.11 and 5.12).

Figs 5.1 and 5.2: Micrographs taken at low magnification of freeze-fracture replicas of adult Nematospiroides dubius. The direction of shadowing is indicated by ⊕ .

Fig. 5.1: The cuticle (C), which is homogeneous and granular in appearance, has partially separated from the epidermal (E) region. The epicuticle shows no sign of fracturing (→). Note the characteristic appearance of the muscle fibres (M).

Fig. 5.2: The outer edge of the cuticle (C) has fractured to reveal a PF face.


M
E
ICE
C
C
ICE
0.5µm
5.1


C
C
ICE
PE
0.5µm
5.2

Where a surface differing from both the P and E fracture faces was observed (Fig. 5.10), it was assumed that the fracture plane had travelled over the nematode exposing the outermost surface. As already indicated, great difficulty was experienced in fracturing the epicuticle of N. dubius. Indeed, in many instances only small areas of the interior of the epicuticle were exposed (Figs 5.3 to 5.5). It was also difficult to locate areas that had been fractured and shadowed at an angle suitable for measuring and counting particles (compare Figs 5.3 to 5.5 with Figs 5.8 and 5.9).

The PF face was covered by a dense array of particles (Figs 5.3 to 5.9). The particle density was 4780 $\pm$ 84 ($\pm$ standard error) per 1 μm^2 with most particles (90.3%) measuring between 6.5 nm and 10.0 nm in diameter. Difficulties arose in defining what constituted a particle because at a magnification of 300 000 times, the particles appeared to be grouped in rows of 2 to 4, while at lower magnifications groups of particles appeared as discrete units (Fig. 5.8 - inset).

The arrangement of particles on the PF face appeared non-uniform (Figs 5.6, 5.8 and 5.9). In some regions, densely packed particles were arranged individually rather than in small groups. These regions extended like furrows between 0.5 µm and 1.75 µm apart (mean = 0.92 $\pm$ 0.36 µm) through the PF face.

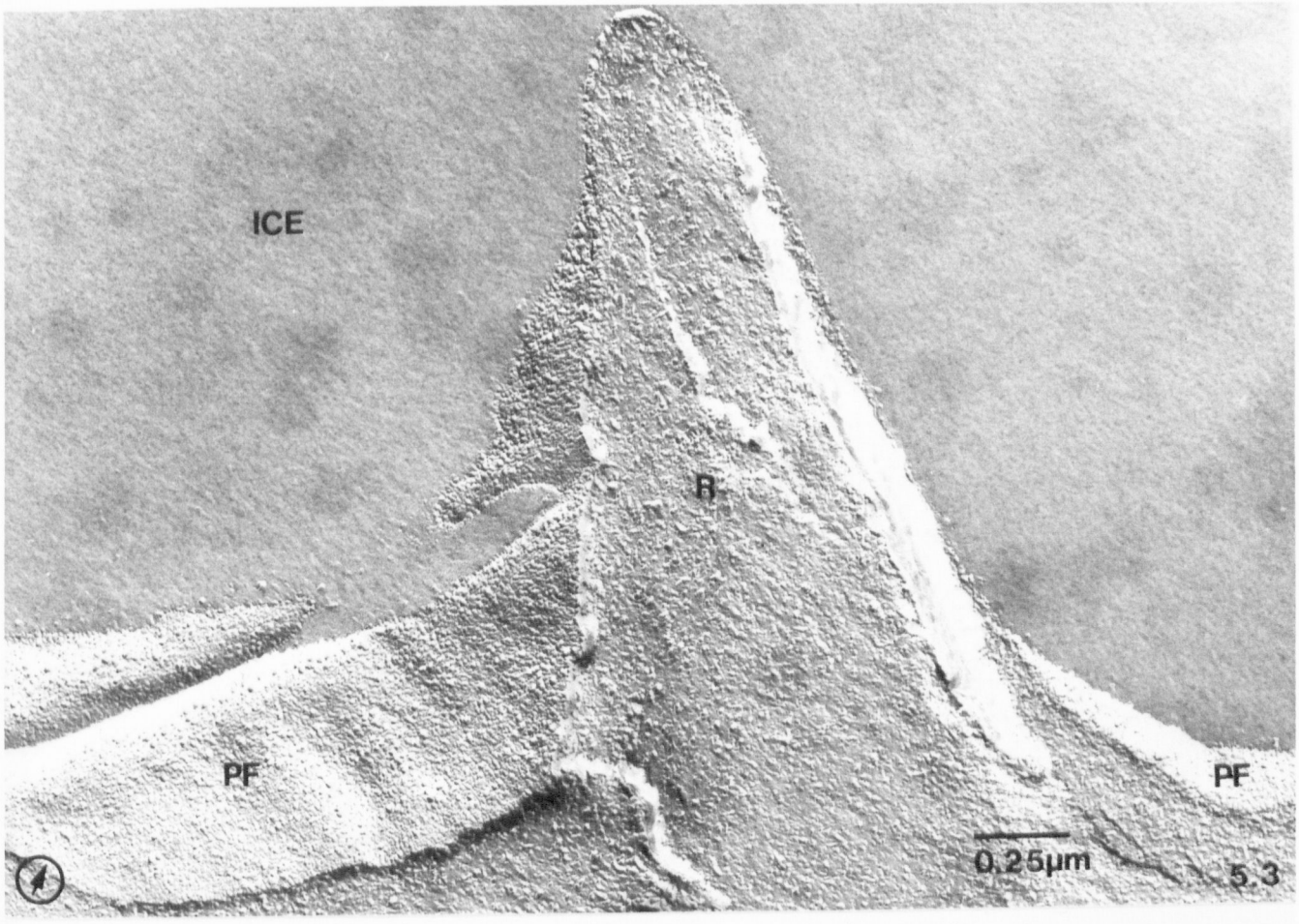
The appearance of the EF face of the epicuticle of adult N. dubius differed markedly from that of the PF face (Figs 5.11 and 5.12). Fewer particles were present with the mean number being 610 $\pm$ 48 ($\pm$ standard error) per μm^2. About 70% of the particles measured 6.5 to 8.0 nm across, while the remainder were about 8 to 10 nm in diameter. Unlike the PF face, the EF face contained about 1850 $\pm$ 59 ($\pm$ standard error) pits per μm^2. Most pits measured between 6.5 and 8.0 nm in diameter, while about 25% measured between 8 and 10 nm across.

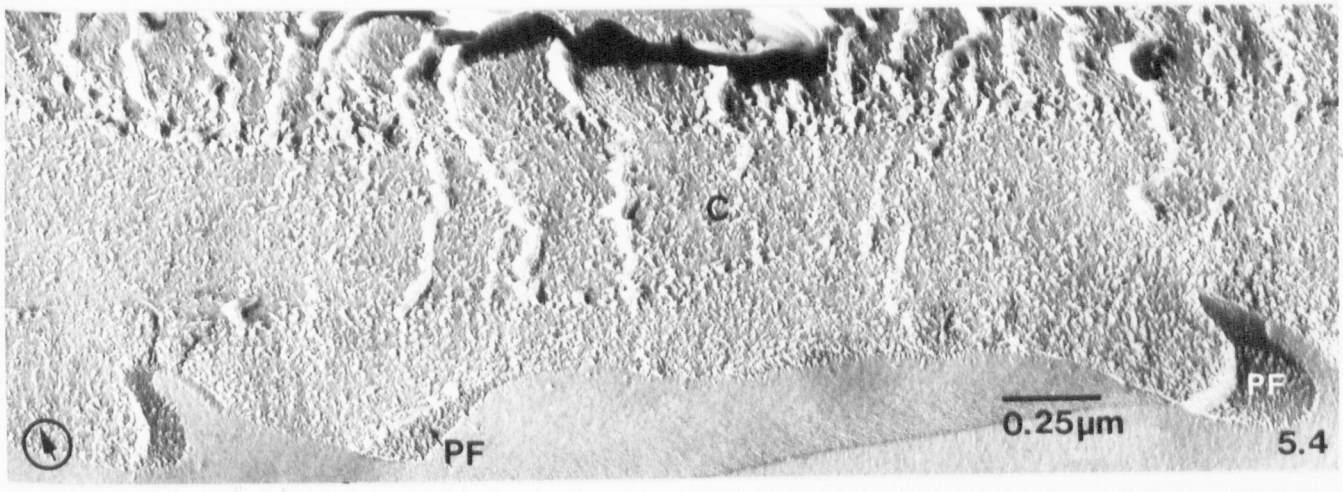
Figs 5.3 to 5.8: Micrographs showing PF faces exposed when the cuticle (C) of adult Nematospiroides dubius was freeze-fractured. The direction of shadowing is indicated by ⊕ .

Fig. 5.3: Areas of the PF face have been exposed on either side of the cuticular ridge (R).

Fig. 5.4: Small areas of the PF face are visible at the outer edge of the cuticle.

Fig. 5.5: The epicuticle has fractured to reveal a PF face unsuitable for counting and measuring particles.


ICE
R
PF
PF
0.25μm
5.3


C
PF
PF
0.25μm
5.4

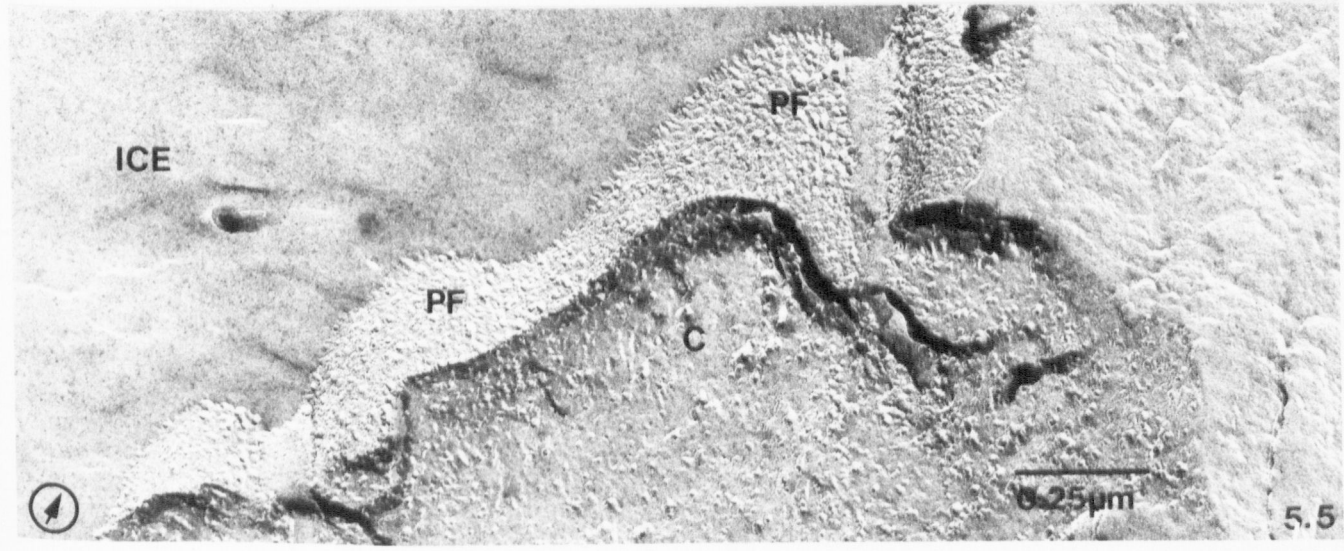
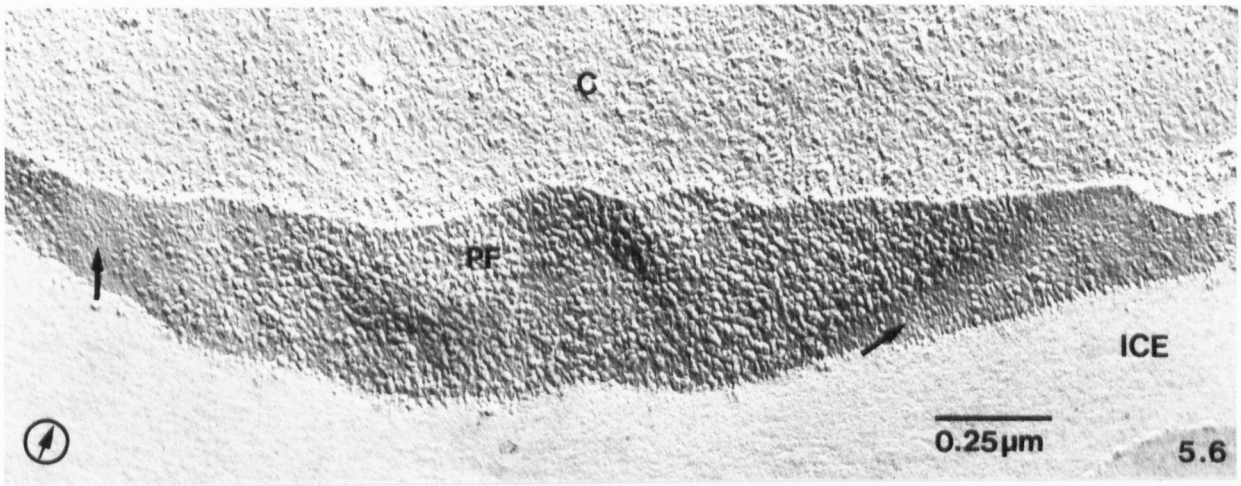
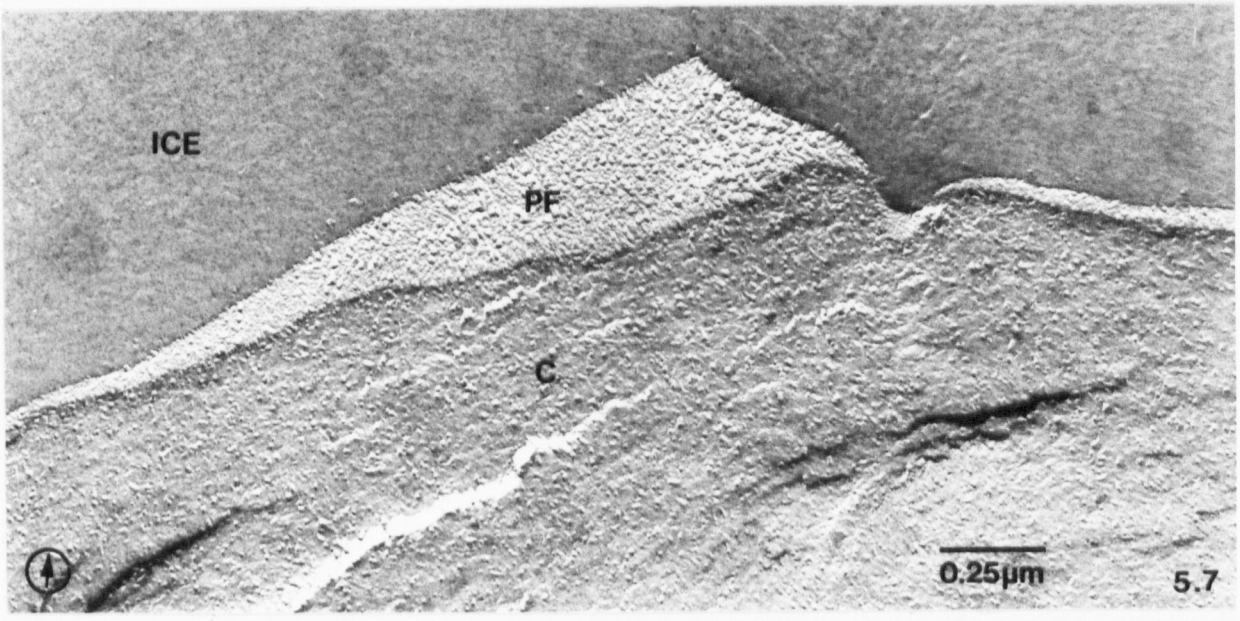

ICE
PF
PF
C
0.25μm
5.5

Fig. 5.6: The arrangement of particles in some regions of the PF face (→) differs from that on the remainder of the exposed face.

Fig. 5.7: A small shelf of the PF face is apparent at the outer edge of the cuticle.

Fig. 5.8: A micrograph depicting the type of fracture face on which particles were measured and counted. Note the particles are arranged in small groups or rows (see inset: (→) x 300 000).


C
PF
ICE
0.25µm
5.6


ICE
PF
C
0.25µm
5.7

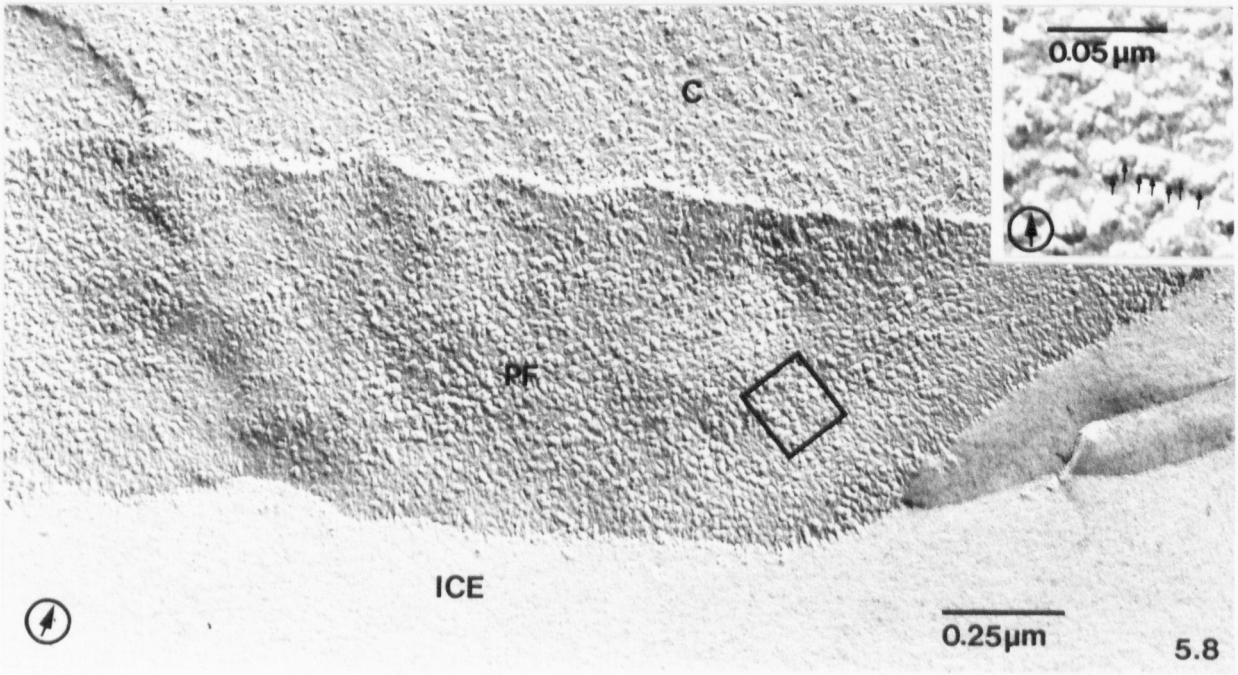
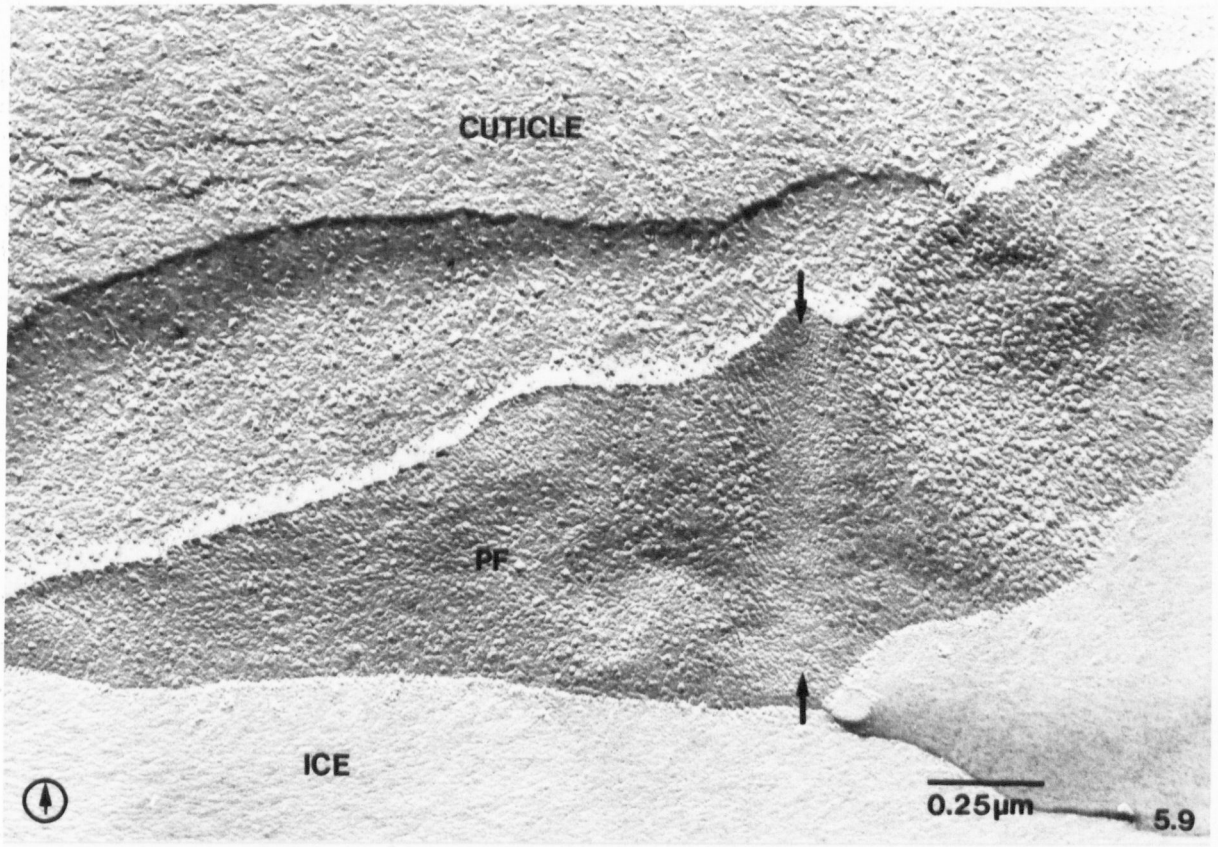
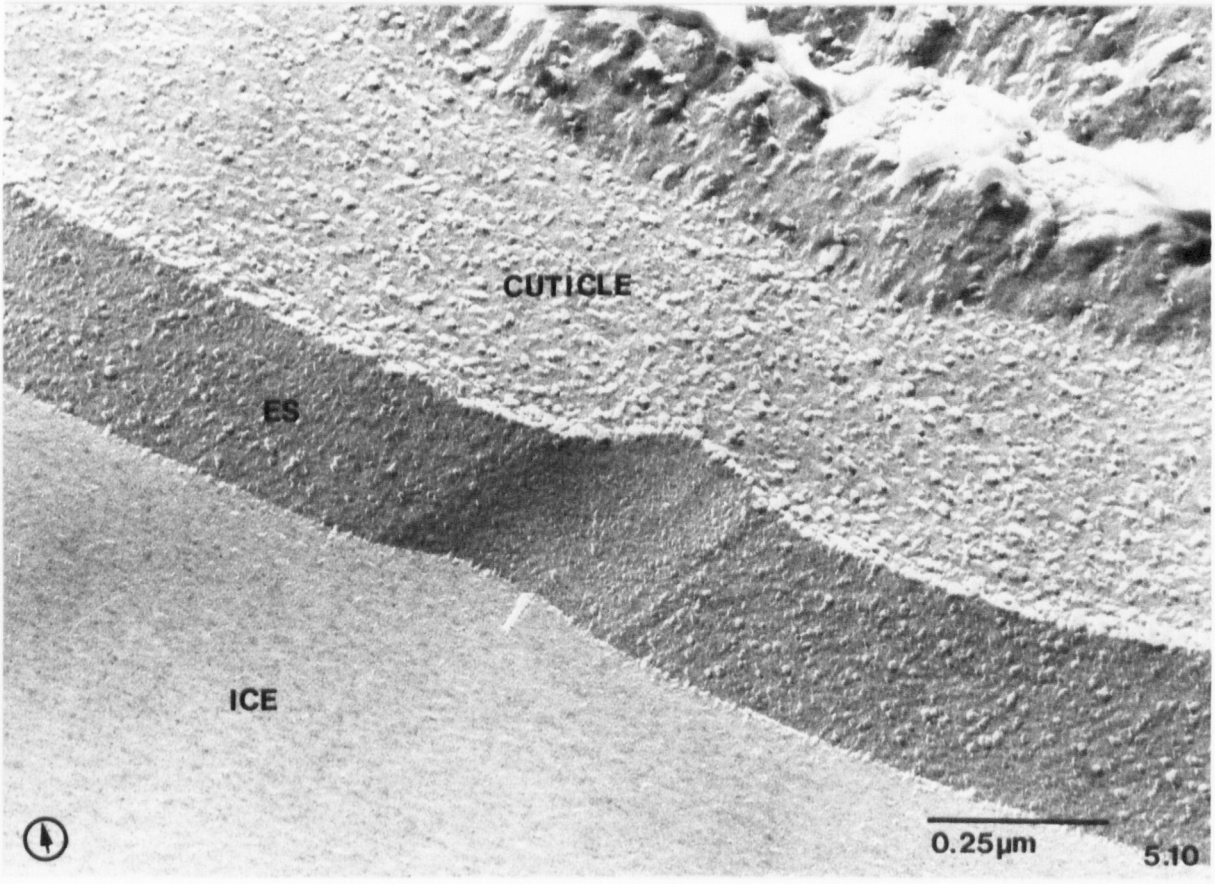

C
PF
ICE
0.25µm
0.05 µm
5.8

Fig. 5.9: Micrograph showing the PF face of the epicuticle of an adult Nematospiroides dubius. Note the groove in which the particles appear smaller (→) compared with the remainder of the PF face. The direction of shadowing is indicated by ⊕ .

Fig. 5.10: The surface (ES) of an adult Nematospiroides dubius exposed when the fracture plane travelled across the outside of the worm. The direction of shadowing is indicated by ⊕ .

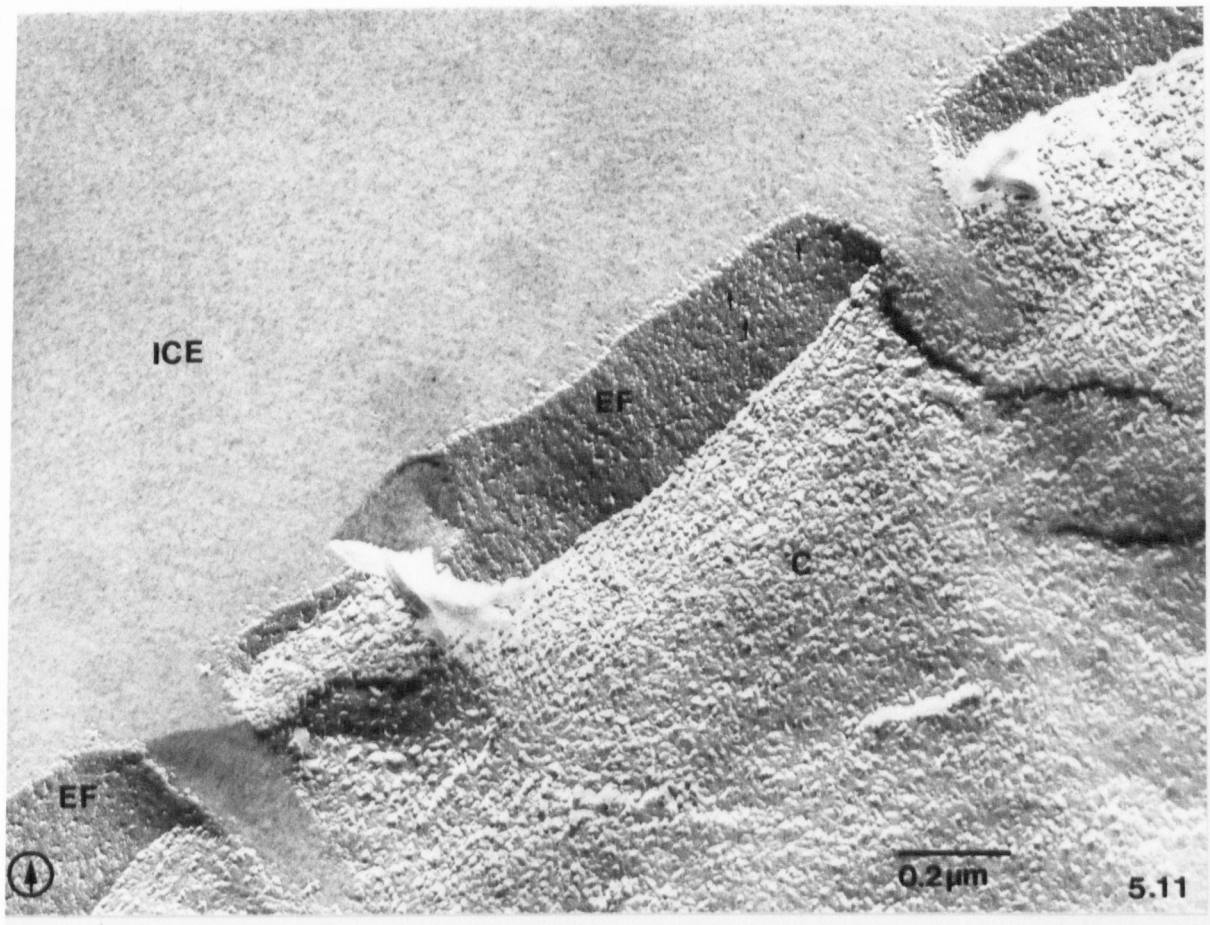

CUTICLE
PF
ICE
0.25µm
5.9


CUTICLE
ES
ICE
0.25µm
5.10

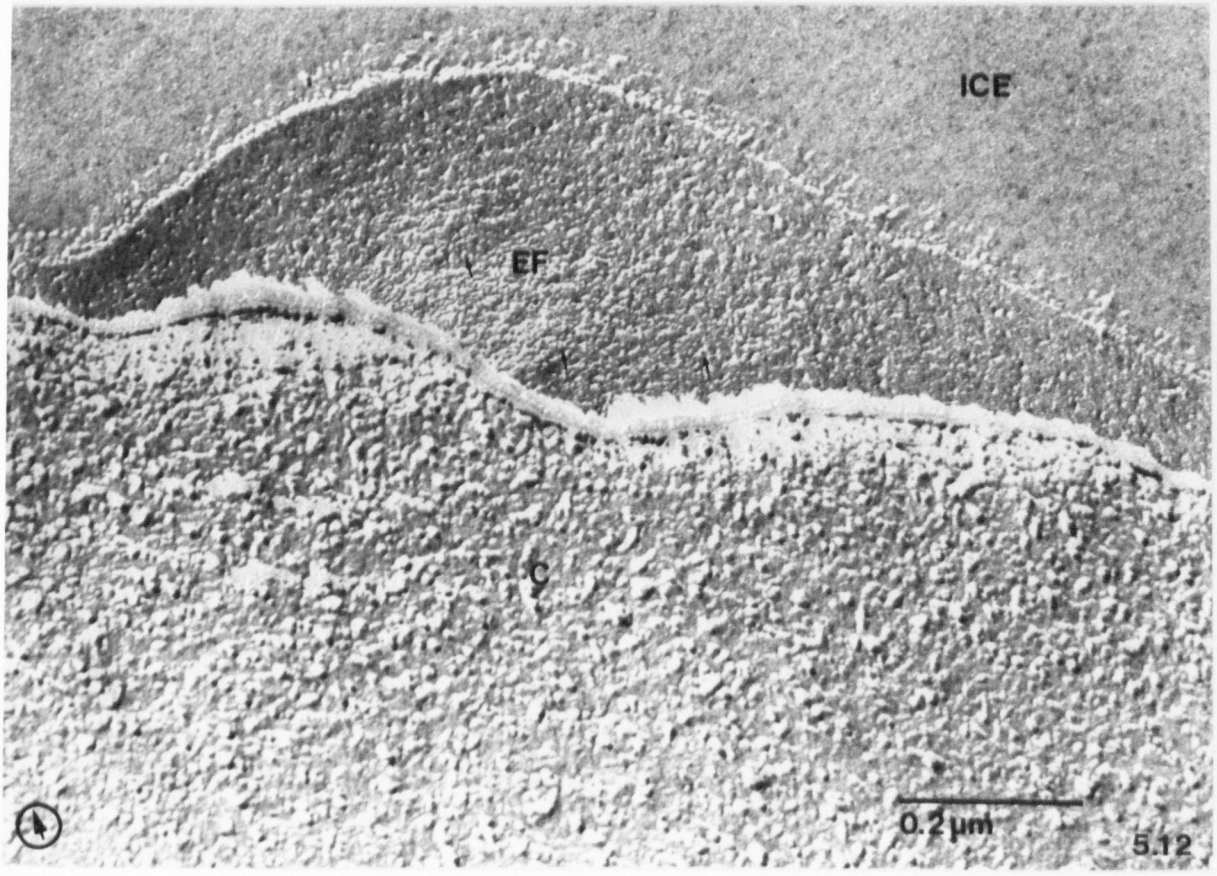
Figs 5.11 and 5.12: Micrographs taken of the freeze-fractured cuticle (C) of adult Nematospiroides dubius in which the EF face is exposed. The direction of shadowing is indicated by ⊕ .

Fig. 5.11: Small regions of the EF face are apparent containing many pits and few particles (→).

Fig. 5.12: The EF face contains few particles (→) but pits are abundant.



5.11



5.12

Thus the epicuticle of adult N. dubius split when freeze-fractured, albeit it with difficulty. In contrast, the epicuticle of third-stage larvae was not seen to cleave in 5 trials. Although the tissue appeared well preserved by the method (Figs 5.13 and 5.14), only cross-fractures were observed.

5.4 DISCUSSION

Because the epicuticle of adult N. dubius cleaved at low temperature, it seems likely that it is largely composed of a lipid bilayer. The presence of particles on the PF face further indicates that the bilayer contains protein complexes. The difficulty in obtaining extensive areas of fractured epicuticle together with the occurrence of numerous pits on the EF face suggests that, like the outer membrane of bacteria, the epicuticle might contain large amounts of lipopolysaccharide. In addition, the high density of particles on the PF face indicates an active role for the epicuticle.

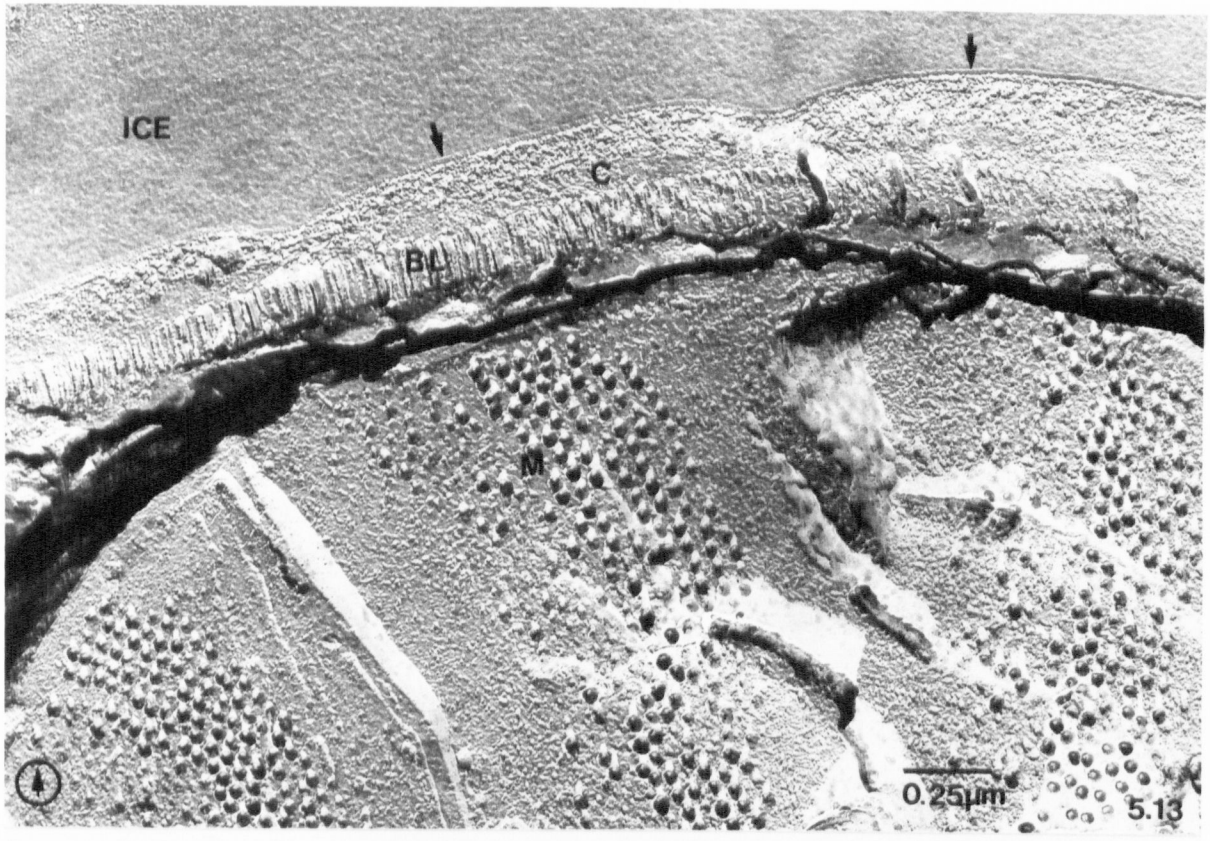
This study also reaffirmed that the composition of the epicuticle of nematodes is variable. The epicuticle of adult N. dubius split when freeze-fractured, but that of third-stage larvae showed a preference to cross-fracture under identical conditions.

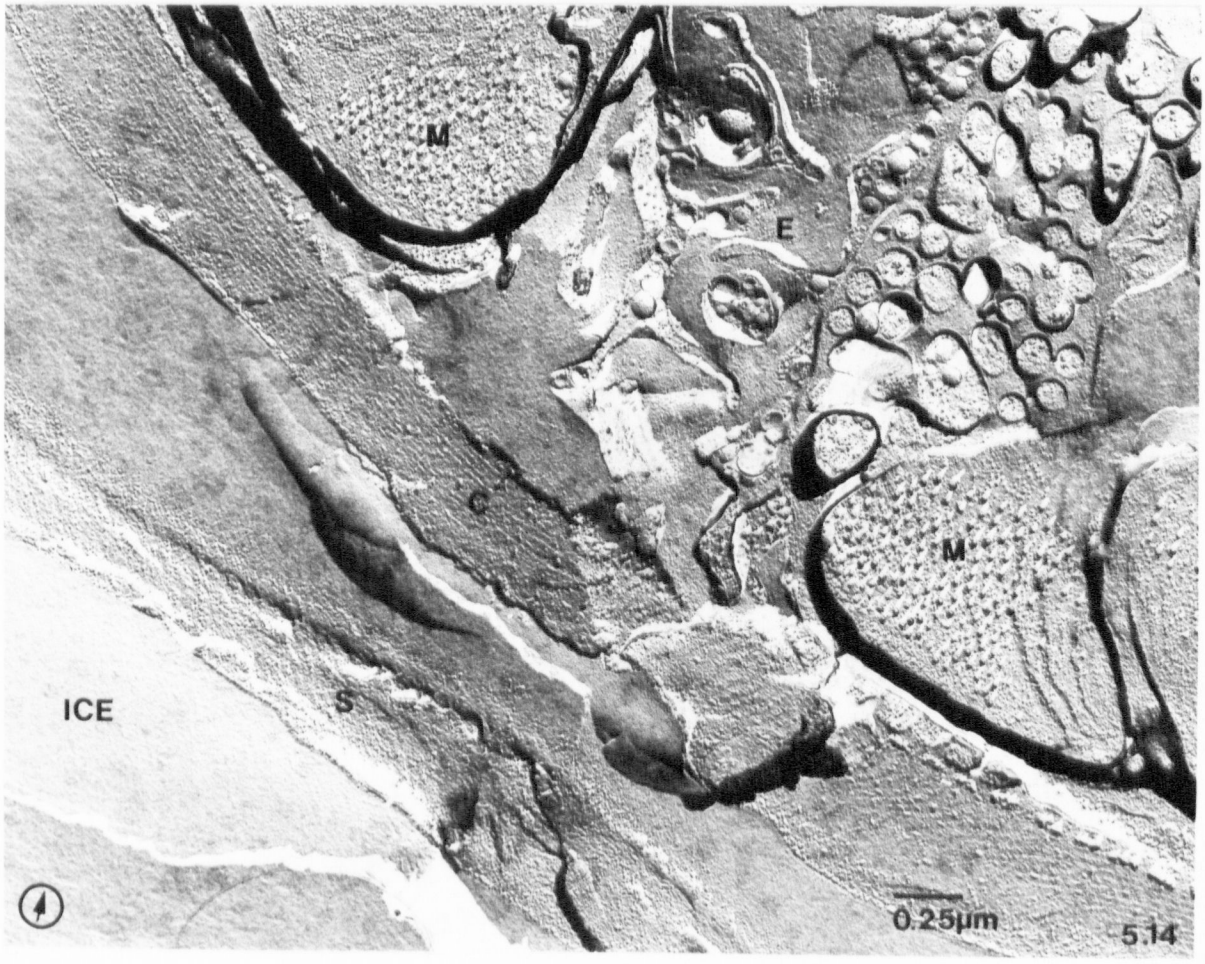
Before discussing variations in the way the epicuticle of different nematodes reacts to freeze-fracturing, the compostion of the epicuticle of adult N. dubius, as revealed by freeze-fracture replication, will be considered in more detail.

Figs 5.13 and 5.14: Micrographs showing cross-fractured third-stage larvae of Nematospiroides dubius. The direction of shadowing is indicated by ⊕ .

Fig. 5.13: The epicuticle (→) is shown cross-fractured. Note the characteristic appearance of the muscle fibres (M) and striated material of the basal zone or layer (BL) of the cuticle (C).

Fig. 5.14: A third-stage larva still within the second-stage sheath (S) was freeze-fractured. Note the characteristic muscle fibres (M) and prominent lateral epidermal region (E). The cuticle (C) has cross-fractured.


ICE
C
BL
M
0.25µm
5.13


M
E
C
M
ICE
S
0.25µm
5.14

5.4.1 Composition of the epicuticle

Using freeze-fracture replication, it appears that the epicuticle of adult N. dubius shares a number of properties with other types of membranes. Like the plasma membrane and some extracellular membranes, such as the bacterial envelope, the epicuticle split through the mid-plane when freeze-fractured to reveal two fracture faces containing particles.

There is little doubt the freeze-fracture line passed through the mid-plane of the adult epicuticle in this study. Examples of both P and E fracture faces were observed. In addition, a surface differing from both PF and EF faces was obtained when the fracture plane passed across the outside of the nematode (see Fig. 5.10). A similar view of the surface of a nematode was obtained by Bird and Buttrose (1974) in a study of the cuticle of A. tritici.

It seems likely that the epicuticle of adult N. dubius is composed largely of a lipid bilayer (consisting of = C18 diglycerides ?) because it split when fractured at low temperature. Evidence indicates that when fractured at low temperature, lipid bilayers tend to split along the plane of lowest energy, namely the terminal methyl groups of the lipid carbon chains, to reveal the interior of the membrane (see Fig. 5.15; Daemer & Branton, 1967; Langworthy, Tornabene & Holzer, 1982).

In contrast, a membrane which is not composed of a lipid bilayer shows a preference to cross-fracture when freeze-fractured (Langworthy et al., 1982; Weiss, 1974). The cell membrane of some Archaebacteria, for example, is composed largely of tetraethers which form a "cross-linked bilayer" (see Fig. 5.15; Langworthy, 1977; Langworthy, 1979). In this membrane, two identical C_{40} - biphytanyl chains extend across the membrane and are attached to glycerol molecules via ether linkages. The cell membrane is in effect made up of a

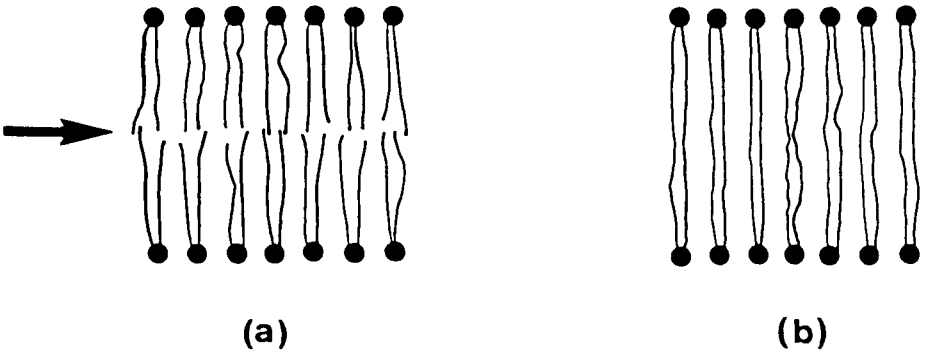


Fig. 5.15: Diagrams depicting (a) the usual lipid bilayer structure and fracture plane (→), and (b) the tetraether "monolayer" lipid membrane found in some Archaebacteria (after Langworthy, Tornabene and Holzer, 1982).

● represent glycerol (polar heads)

represent (a) variable length hydrocarbon chains of phospholipids
(b) C_{40} - biphytanyl chains

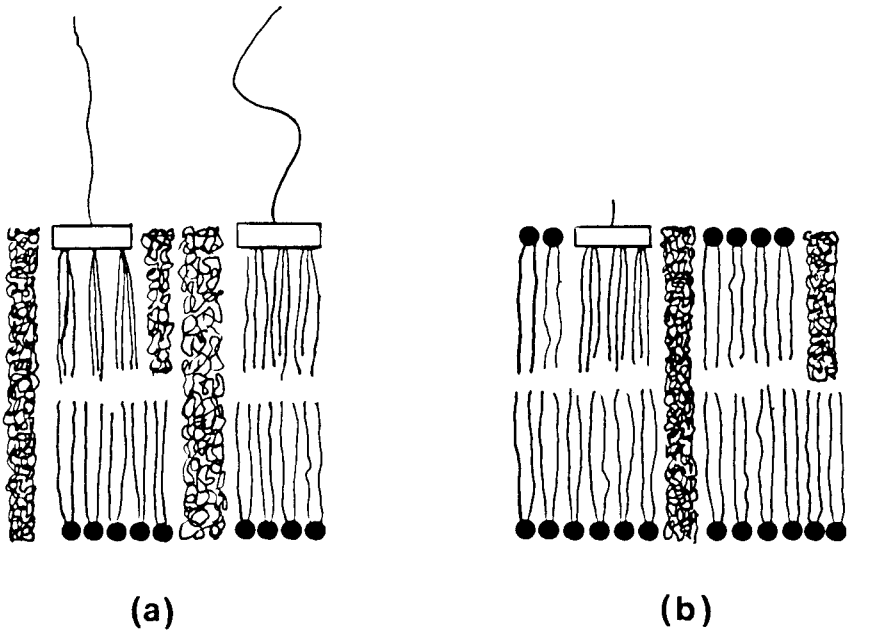


Fig. 5.16: Diagrams depicting the structure of the outer membrane of Escherichia coli and Salmonella typhimurium (a) Wild-type strains, and (b) "deep-rough" mutants (after Nikaido, 1979).

represent proteins

represent phospholipids

represent lipopolysaccharides

monolayer of amphiphilic lipid molecules. Because the lipid molecules are condensed at the centre, they do not separate to reveal the interior of the membrane when freeze-fractured (Ververgaert, pers. comm. in Langworthy, 1979; Weiss, 1974).

The importance of lipid in the bilayer structure of membranes has also been demonstrated using organic solvents. When lipid is extracted from a plasma membrane or the outer membrane of bacteria using acetone or chloroform-methanol, the planes of weakness along which the membranes usually split when freeze-fractured disappear, and the membrane breaks at random (Branton & Park, 1967; Forge & Costerton, 1973).

Thus for a membrane to split when freeze-fractured, it must be largely composed of a bilayer of lipid. The epicuticle of adult N. dubius split to reveal two fracture faces when freeze-fractured, and so it seems likely that a lipid bilayer forms an important part of its structure.

There were, however, a number of unusual features associated with fracturing the epicuticle of N. dubius which indicate that the epicuticle may contain a high proportion of lipopolysaccharides, such as is found in the envelope of Gram-negative bacteria (Bayer et al., 1975; Bayer, 1981; Beveridge, 1981; Nikaido, 1979; Salton & Owen, 1976, Smit et al., 1975). Like the outer membrane of Wild-type strains of E. coli and Salmonella typhimurium, the epicuticle of N. dubius was difficult to fracture, and once fractured, one of the fracture faces appeared highly pitted. (It should be noted that it is the PF face of the bacterial envelope which appears pitted, whereas the EF face of the epicuticle of N. dubius contained depressions).

The difficulty in fracturing the outer membrane of bacteria has been attributed with the presence of only small amounts of phospholipid compared with protein and lipopolysaccharide. Where the outer membrane

contains a heptose-deficient lipopolysaccharide, such as in "deep rough" mutants, it freeze-fractures more readily (Bayer et al., 1975; Irvin, Chatterjee, Sanderson & Costerton, 1975, Smit et al., 1975). This increased tendency to cleave through the mid-plane is related to the increased ratio of phospholipid to protein of the membrane (Fig. 5.16; Bayer et al., 1975).

Not only does the epicuticle of adult N. dubius share freeze-fracturing characteristics with the outer membrane of some Gram-negative bacteria, such as E. coli and S. typhimurium, but the two organisms occupy the a similar environment. Both are found in the intestinal tract of higher animals and so are subject to hydrophobic inhibitors, such as bile salts. To survive, these organisms must be able to keep out noxious compounds which easily penetrate most biological membranes.

The capacity of bacteria to withstand the enteric environment has been attributed to a high proportion of lipopolysaccharide within the outer leaflet of the outer membrane (see Fig. 5.16; Nikaido, 1979). These lipopolysaccharides interact with proteins to form a barrier; hydrophobic molecules cannot penetrate the hydrophilic portion of the protein molecules on the surface of the membrane, nor can they penetrate the highly charged hydrophilic core of the lipopolysaccharide molecules.

It is tempting to speculate that, like the outer membrane of bacteria, the epicuticle of adult N. dubius contains a high proportion of lipopolysaccharides which renders it relatively impermeable to hydrophobic compounds (see Chapter 6). A high proportion of lipopolysaccharide would also make the epicuticle difficult to freeze-fracture.

That the epicuticle of N. dubius has an active metabolic role is indicated by the dense array of particles on the PF face. The particles

seen on other biological membranes have been shown to consist of protein or aggregates of protein molecules so it seem plausible that the particles present on the PF face of N. dubius also represent proteins (McNutt, 1977; Zingsheim & Plattner, 1976).

Similar particles were detected on the PF face of the epicuticle of second-stage M. javanica (Bird, 1984). Although the number of particles present on the PF face of M. javanica were not estimated, the IMP's were descibed as being "quite densely packed", a description in keeping with the average of 4800 particles per μm^2 counted in this study (Bird, 1984).

Membranes engaged in synthetic activity generally contain a large number of IMP's. The PF face of the highly active thylakoid membrane, for example, contains some 6200 particles per μm^2 (Simpson, 1979). About 1100 particles per μm^2 were found on the PF face of a mature spermatozoan of N. brasiliensis taken from the seminal vesicles, and this number increased to about 3400 particles per μm^2 following activation (Wright, Cox & Dwarte, 1985).

The role of the particles on the PF face of the epicutile is not known, but it is possible that the particles represent transport proteins involved in maintaining the impermeability of the epicuticle. Transport proteins actively pump ions and other molecules against a concentration gradient so making biological membranes impermeable. Moreover, because transport proteins are large molecules, they represent the proteins most likely to be detected by freeze-fracture replication (Alberts et al., 1983).

Fewer particles were present on the EF face of the epicuticle of N. dubius compared with the PF face. Unfortunately Bird (1984) did not include a micrograph of the EF face of epicuticle of M. javanica so no comparisons can be made. Indeed no freeze-fracture study of the

nematode epicuticle, to my knowledge, has included a view of the EF face of the epicuticle. In this study, there were more than twice the number of particles on the PF face than pits on the EF face. Some of the smaller pits, of course, could have been obscured by the 2 nm thick coating of platinum used to shadow the replicas. But a lack of complementarity between P and E fracture faces occurs commonly in other fractured membranes (Flower, 1973; Sletyr & Robards, 1982; Zingsheim & Plattner, 1976).

In some regions of the PF face of N. dubius, particles were arranged in a different way to the rest of the PF face (see Figs 5.7 to 5.9). These regions appeared to correspond to the presence of annulations along the length of the worm (mean distance between annulations = 1.24 $\pm$ 0.19 µm [$\pm$ standard error, measurements taken from 6 micrographs] compared with the mean distance between longitudinal ridges (10.54 $\pm$ 1.27 µm [$\pm$ standard error, 20 measurements from 4 thick sections]).

When discussing the capacity of the epicuticle to freeze-fracture, the difficulties experienced in using the technique must be mentioned. Particles could not be counted or measured on many of the fracture faces because the angle of fracture and shadow was unsuitable. Consequently only limited areas of both P and E fracture faces could be used for quantifying particle size and number.

In addition, it was difficult to identify exactly what constituted a particle. Some particles appeared to be made up of two or three smaller ones which would, of course, affect the number and size of the particles counted. However the size of the particles measured in this study (6.5 and 10.0 nm) was similar (8 and 10 nm) to those measured by Bird (1984). Similar problems were experienced when counting pits on the EF face. Although two counts were made over each area, in an effort

to compensate for these uncertainties, the problems must be kept in mind.

Despite the difficulties associated with recognizing and counting the IMP's, it appears that the epicuticle of adult N. dubius is composed largely of a bilayer of lipid, a substantial proportion of which might be lipopolysaccharides or lipopolysaccharide-protein complexes. The presence of particles on the fracture faces suggests that the epicuticle might contain protein and could be metabolically active.

5.4.2 Variations in the epicuticle

To conclude that all nematodes are bounded by a biological membrane, as described for adult N. dubius, would be too simplistic an explanation. Differences in the way that the epicuticle of nematodes reacts to freeze-fracturing have been recorded between species, and in this study, between two stages of one species (Bird, 1984; Lee et al., 1984; Martinez-Palomo, 1978). No other study, to my knowledge, has sought to compare the freeze-fracture characteristics of cuticles from different stages of one species.

As already discussed, the epicuticle of adult N. dubius, which split when freeze-fractured, appears to be composed largely of a lipid bilayer which contains protein and lipopolysaccharide (see Section 5.4.1). In contrast, the third-stage epicuticle did not split when freeze-fractured; instead only cross-fractures were observed. Of course, the failure of the third-stage cuticle to fracture does not prove that the third-stage epicuticle of N. dubius is not a bilayer. Rather it suggests that third-stage and adult epicuticles differ in their composition. Ultrastructural studies support this conclusion (see Figs 3.11 and 3.14).

The appearance of the PF face of N. dubius obtained in this study is similar to that found in M. javanica (Bird, 1984). In both cases the

PF face was densely studded with particles. The EF face of N. dubius, on the other hand, contained few particles but was highly pitted. There are no published micrographs of the EF face of a nematode epicuticle so it is not known whether a pitted EF face is a common feature of the nematode epicuticle.

An interesting comparison can be made, however, between the the epicuticle of N. dubius and the multilaminate surface of the adult flukes (Belton & Belton, 1971; Hockley, McLaren, Ward & Nermut, 1975). The surface of Schistosoma mansoni, appears to be bounded by two membranes arranged back to back, each of which fractures in a similar way to a plasma membrane. The inner- and outer-most faces exhibit IMP's, while the two inner faces contain fewer particles but have many pits. The individual components of the surface of S. mansoni resemble the epicuticle of N. dubius and other cell membranes, but like some Gram-negative bacteria, S. mansoni presents an outer membrane leaflet, complete with particles, to the environment.

Although the appearance of the PF face of N. dubius and M. javanica is similar, results obtained for other nematodes indicate that the compostion of the epicuticle of nematodes may have fundamental variations. Like N. dubius and M. javanica, the epicuticle of N. brasiliensis also split when freeze-fractured (Lee & Bonner, 1982). But because only minute particles were located on the fracture faces, the workers expressed reservations as to whether the particles were true IMP's.

Where freeze-fracture replication has been used to study epicuticles which are multilaminate, some interesting results have been obtained. In microfilariae of O. volvulus, a fracture plane passed between two inner layers of the epicuticle (the second and third layers, using the terminology of Marinez-Palomo, 1978). Large areas of the

interior of the epicuticle were exposed which appeared quite smooth. It should be noted, however, that each of the dense layers making up the epicuticle of O. volvulus was composed of a trilaminate structure and the fracture plane passed between two trilaminate structures rather than through the mid-plane of any of them.

The structure of the material lying at the surface of the first-stage larvae of T. spiralis is interesting. Although the surface of the cuticle did not split when freeze-fractured, a layered structure which covers the cuticle did fracture (Lee et al., 1984). The outer layer of this surface structure was found to consist of particles embedded in an amorphous matrix, while the inner layer was apparently composed of a thin sheet of parallel filaments or ridges of fused particles which gave it a striated appearance.

The striated appearance of layer 3 (using the nomenclature of Lee et al., 1984) resembles the pellicle, or cell membrane complex, of Euglena gracilis var. bacillaris when freeze-fractured (Miller & Miller, 1978). The cortical region of the pellicle complex of E. gracilis freeze-fractures to reveal a striated PF face which is complementary to the EF face. Complementarity of P and E fracture faces was only achieved where E. gracilis was fractured when the temperature was less than -150°C which demonstrates that the techniques used in freeze-fracturing are important in determining the results. Caution is needed when comparing the results of freeze-fracture studies.

In this study, however, two stages of N. dubius were freeze-fractured under identical conditions, and the epicuticle reacted in different ways. It seems likely that the epicuticle of these two stages, at least, differs in composition.

CHAPTER 6

COMPOSITION OF THE EPICUTICLE OF N. dubius

6.1 INTRODUCTION

The cuticle of nematodes appears to be an extracellular structure which forms from molecules secreted from the epidermis (see Chapter 4; Bonner & Weinstein, 1972a; Martinez-Palomo, 1978). A large proportion of the inner region of the cuticle is composed of collagen-like proteins (Anya, 1966; Cox et al., 1981a; 1981b; Evans et al., 1976; Josse & Harrington, 1964; Leushner et al., 1979; McBride & Harrington, 1976a; 1976b; Noble et al., 1978) a feature it shares with other extracellular structures (see Chapter 1; Hay, 1981).

The composition of the epicuticle is rather different from that of the inner cuticle (Cox et al., 1981a; Fujimoto & Kanaya, 1973; Fauré-Fremiét & Garrault, 1944). But it too appears to contain molecules that are similar to those found in other envelopes. The freeze-fracturing characteristics of the epicuticle of adult N. dubius, for example, are similar to those of Gram-negative bacteria (see Chapter 5; Irvin et al., 1975; Smit et al., 1975).

Studies involving freeze-fracture replication suggest that an important component of the epicuticle of some nematodes might be a bilayer of lipid; phospholipids probably do not predominate (see Chapter 5; Bird, 1984; Lee & Bonner, 1982). In addition, carbohydrate is associated with the external surface of the epicuticle (McClure & Zuckerman, 1982; Nordbring-Herz & Mattiasson, 1979; Sneller et al., 1981; Spiegel et al., 1982; Spiegel et al., 1983; Zuckerman et al., 1979; Zuckerman & Kahane, 1983). Some of the carbohydrate, at

least in T. spiralis and T. semipenetrans, appears to be conjugated to protein in the form of glycoprotein (Clark et al., 1982, Spiegel et al., 1982). Furthermore, in most of the free-living and parasitic nematodes that have been studied, the surface exhibits a net negative charge due, in part, to sulphate groups (Himmelhoch et al., 1977; Himmelhoch et al., 1979; Hudson & Kitts, 1971; Murrell et al., 1983).

In this chapter, I will give the results of a study of the surface of N. dubius which used a variety of common stains and solvents. Of particular interest was the composition of the adult epicuticle, but differences between this and the third-stage epicuticle were also examined. Some properties of the surfaces of first-, second- and fourth-stage larvae were also briefly considered.

6.2 MATERIALS AND METHODS

Unless otherwise stated, stains were used on whole worms because preservation of the internal structure of the worm was not as critical as the effect of the stain on the surface of the worm. N. dubius was collected as described in Sections 2.2.2 and 2.2.3, and adults were cut into pieces in the first change of absolute ethanol prior to embedding (see Section 2.3).

A variety of reagents were used (see Table 6.1 for a list of reagents and their properties), and the effect they had on the surface on N. dubius was assessed using transmission electron microscopy (see Section 2.3). At least 5 worms were examined from each treatment.

6.2.1 Cationized ferritin

A few drops of cationized ferritin (10.4 mg per ml; Sigma Chemical

REAGENTS	PROPERTIES	REFERENCES
Cationized ferritin	Binds non-specifically to negative charge.	Danon *et al.* (1972)
Ruthenium red	Binds to polyanions via electro-static bonds but also reacts with lipid. Used to demonstrate cell surface material.	Blanquet (1976a) Blanquet (1976b) Dierichs (1979) Luft (1971)
Alcian blue-lanthanum nitrate	Binds to negative charge at neutral pH. Enhances cell coat preservation.	Shea (1971) Shea & Karnovsky (1969)
Tannic acid (gallotannin)	Acts primarily as a mordant between osmium tetroxide and lead stains. Cross-links protein and enhances membrane contrast.	Gustavsen (1949) Kalina & Pease (1977) Simionescu & Simionescu (1976a; b) Wagner (1979)
Cuprolinic blue	Stains proteoglycan where tissue is fixed with glutar-aldehyde or paraformaldehyde.	Scott (1972) Scott *et al.* (1981) Scott (1980)
Periodic acid bismuth sub-nitrate	Localization of periodate reactive mucosubstances and polysaccarides containing 1,2-glycols in thin sections.	Ainsworth *et al.* (1972)
Concanavalin A	Binds specifically to α-D-mannoside or α-D-glucoside residues on a variety of oligosaccharides.	Nicholson (1974) Lis & Sharon (1977) Sharon & Lis (1975)

Table 6.1: List of reagents used to probe the surface of *Nematospiroides dubius* (Table 6.1 continues...see over).

REAGENTS	PROPERTIES	REFERENCES
Wheat germ agglutinin	Binds to N-acetyl-D-glucosamines, N-acetyl-D-neuraminic acid and N-acetyl-D-galactosamine.	Jordan et al. (1977) Sharon & Lis (1975) Lis & Sharon (1977)
Sodium dodecyl sulphate	Anionic detergent which binds to protein via hydrophobic interactions and dissociates membranes.	Quinn (1976) Gel'man et al. (1975)
β-mercapto-ethanol	Reducing agent that prevents aggregation of protein by forming intermolecular S-S bonds.	Lis & Sharon (1977)
Elastase (EC. 3.4.21.36)	Cleaves bonds involving the carboxyl groups of Glycine, Alanine, L-Leucine, L-Valine and L-Isoleucine	Enzyme Nomenclature-1984
Hyaluronidase (EC. 3.2.1.35)	Randomly hydrolyzes 1,4-linkages between N-acetyl-β-D-glucosamine and D-glucuronate residues in hyaluronate. Also hydrolyzes 1,4-β-D-glycosidic linkages between N-acetyl-galactosamine or N-acetyl-galactosamine sulphate and glucoronic acid in chondroitin, chondroitin 4- and 6-sulphate and dermatan.	Enzyme Nomenclature-1984
Neuraminidase (=Sialidase) (EC. 3.2.1.18)	Hydrolysis of 2,3- 2,6- & 2,8-glucosidic linkages between terminal non-reducing N- or O-acylneuraminyl residues and galactose N-acetylhexosamine, or N- or O-acylated neuraminyl residues in oligo-saccharides, glycoproteins, glycolipid or colominic acid.	Enzyme Nomenclature-1984

Table 6.1 (...continued): List of reagents used to probe the surface of Nematospiroides dubius.

REAGENTS	PROPERTIES	REFERENCES
Sodium hypochlorite	Oxidizing agent of unknown mechanism but it is thought to combine with protein.	Dychdala (1983)
Guanidine-HCl	Dissolves certain proteins by preventing hydrogen bonding and denaturing secondary and tertiary structures.	Quinn (1976)
Aqueous phenol	Dissolves protein, lipopolysaccharide, lipoprotein and glycoprotein. Dissolves the outer membrane of bacteria.	Bladen & Mergenhagen (1964) Westphal & Jann (1965)
Formic acid	Acts with H-bonds to fragment membranes.	Zahler & Niggli (1977)
Chloroethanol-sulphuric acid	2-chloroethanol dissolves protein and together with mineral acids, dissolves chitin.	Muzzarelli (1977) Zahler & Niggli (1977)
n-Butanol	Solubilizes membrane lipid and protein when used with water.	Zahler & Niggli (1977)
Diethyl ether	Extracts lipid from biological material.	Zahler & Niggli (1977)
Chloroform: methanol	Extracts lipid from biological material.	Zahler & Niggli (1977)
Sodium periodate	The periodate ion is thought to degrade proteoglycan and polysaccharide of connective tissue.	Scott et al. (1972) Scott et al. (1976)

Table 6.1 (...continued): List of reagents used to probe the surface of Nematospiroides dubius.

23., USA) were added to first-, second-, third- and fourth-stage larvae, and adult worms suspended in a drop of phosphate-buffered saline or Sörensen's phosphate buffer, pH 7.2 at 22°C (Danon, Goldstein, Marikovsky & Skutelsky, 1972). Worms were agitated for 5 to 10 minutes, and then washed 3 times in the appropriate buffer before being processed for transmission electron microscopy (see Section 2.3).

Control adult worms were pre-treated either with poly-L-lysine (10 mg per ml, MW 90 000; Sigma Chemical Co., USA) in phosphate-buffered saline pH 7.2, for 1 hour at 22°C, or with neuraminidase (EC 3.2.1.18, 10 units per ml; Type V, Sigma Chemical Co., USA) in 0.05M sodium acetate pH 5.5 containing 0.85% sodium chloride for 2 hours at 37°C (Gress & Lumsden, 1976a). Worms were then washed in 3 changes of the appropriate buffer and labelled with cationized ferritin.

An attempt was made to quantify the amount of cationized ferritin binding to the surface of adults using microprobe analysis (ap Gwynn, 1981). Initial studies using an uneven surface, sputter-coated with iron and varying amounts of copper, indicated that an estimate of the amount of iron present could be obtained (see Appendix 1). Adult worms, labelled with cationized ferritin, were briefly fixed in 3% glutaraldehyde and laid out straight between 2 glass slides at 37°C overnight. One slide was removed and the worms were sputter-coated with copper.

Counts were made over an area of about 0.045 mm^2 for 60 seconds using a JEOL 733 scanning electron microscope analyzer, with KEVEX energy dispersive X-ray detector at a beam current of 0.84 nA and an accelerating voltage of 15kV. Eight energy "windows" were analyzed: silicon (1700 - 1800 eV), phosphorous (1960 - 2060 eV), molybdenum (2260 - 2360 eV), chloride (2580 - 2650 eV), potassium (3280 - 3380 eV), background (6040 - 6140 eV), iron (6360 - 6460 eV) and background

(6740 - 6840 eV). The set counts for iron, and therefore cationized ferritin, were obtained by subtracting the counts for the background from the counts collected from the energy "window" for iron.

6.2.2 Ruthenium red

Adults, and exsheathed and ensheathed third-stage larvae, were stained with ruthenium red (Taab Laboratories Equipment Ltd., England) following the method of Luft (1971). Exsheathed larvae were isolated from the stomach of a mouse 30 minutes after infection (see Section 2.2.2).

Worms were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2, containing 0.1% ruthenium red for 1 hour at 22°C, and then rinsed in 3 changes of the same buffer over 15 minutes. Tissue was post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate pH 7.2, containing 0.1% ruthenium red for 3 hours at 22°C in the dark because the osmium tetroxide-ruthenium red mixture is unstable in light (Hayat, 1981). Following several rinses in 0.1M sodium cacodylate buffer, the tissue was dehydrated and embedded as described in Section 2.3.

It should be noted that some infective stages were still active after 1 hour in the glutaraldehyde staining solution, but most stopped moving when washed in buffer prior to fixation in the osmium tetroxide staining solution.

Control worms were pre-treated with neuraminidase (10 units per ml; Type V, Sigma Chemical Co., USA) in 0.05M sodium acetate buffer pH 5.5, for 2 hours at 37°C (Gress & Lumsden, 1976a).

6.2.3 Alcian blue-lanthanum nitrate

Adult worms were fixed in 3% glutaraldehyde in 0.1M sodium

cacodylate buffer pH 7.2, containing 0.5% alcian blue (Difco, UK; 8007-13, LINO 74240, control 1920) for 2 hours at 22°C (Shea, 1971). They were washed briefly in the same buffer and transferred to 1% osmium tetroxide in 0.1M sodium cacodylate buffer pH 7.2, containing 1% (w/v) lanthanum nitrate (Ajax Chemicals, Australia) for 2 hours at 22°C (Cherian et al., 1980; Hayat, 1981; Zalik, Sanders & Tilley, 1972). Caution was necessary because lanthanum ions precipitate at low temperature and in the presence of phosphate buffer (Hayat, 1981). Following a brief rinse in 0.1M sodium cacodylate buffer pH 7.2, worms were dehydrated and embedded as described in Section 2.3. Sections were viewed without counter-staining.

6.2.4 Tannic acid

Tannic acid (A. R. Code no. 1764, Mallinckrodt Inc., USA) was used as a stain, and a as fixative, on adults and exsheathed third-stage larvae of N. dubius.

Worms to be stained with tannic acid, following the method of Simionescu and Simionescu (1976a), were fixed as described in Section 2.3, except that 0.1M sodium cacodylate buffer pH 7.2, was used instead of Sörensen's phosphate buffer. Following fixation, worms were washed thoroughly in 0.1M sodium cacodylate buffer pH 7.2, at 22°C over 15 minutes because osmium tetroxide reacts with tannic acid (Hayat, 1981). Staining was carried out in 1% tannic acid in 0.05M sodium cacodylate buffer pH 7.0, for 30 minutes at 22°C. Excess tannic acid was removed by washing in 1% sodium sulphate (BDH Lab. Chemicals, England) in the same buffer for 5 minutes. Worms were dehydrated and embedded as described in Section 2.3.

When tannic acid was used as a fixative (Locke & Huie, 1975; Locke & Huie, 1980), worms were fixed in 3% glutaraldehyde in

0.1M sodium cacodyate buffer pH 7.2, containing 2.5% tannic acid (which had been pre-neutralized with dilute sodium hydroxide) at 0°C. They were rinsed for 30 minutes in cold 0.1M sodium cacodylate buffer pH 7.2, and post-fixed in osmium tetroxide in the same buffer at 0°C. Because sodium cacodylate and uranyl salts are not compatible (Hayat, 1981), tissue was washed in distilled water before being incubated in 2% aqueous uranyl acetate in the dark for either 6 or 20 hours at 55°C. After a brief rinse in distilled water, worms were dehydrated and embedded as described in Section 2.3. Sections were counter-stained for 3 or 5 minutes with lead citrate (Reynolds, 1963).

6.2.5 Cuprolinic blue

Ensheathed and exsheathed third-stage larvae of N. dubius, as well as adult worms, were stained in cuprolinic blue (BDH Lab. Chemicals, England) following the method of Scott, Orford and Hughes (1981).

Adult worms were fixed for 15 minutes in 2.5% glutaraldehyde in 0.025M sodium acetate buffer pH 5.7, at 22°C and then cut into small pieces. Ensheathed third-stage worms were cut in half in the same fixative. The tissue was stained in 0.05% cuprolinic blue in 0.025M sodium acetate buffer pH 5.7, containing 0.1M magnesium chloride and 2.5% glutaraldehyde for 20 hours at 22°C. Tendons from the tail of an adult mouse, teased into 0.9% saline, were fixed as a positive control. Negative controls were fixed in the same solution except that the dye was omitted.

Following staining, the tissue was rinsed free of cuprolinic blue using 0.025M sodium acetate buffer pH 5.7, containing 2.5% glutaraldehyde and 0.1M magnesium chloride over 30 minutes, and post-stained in 0.5% aqueous sodium tungstate (tungstic acid; Sigma Chemical Co., USA) for 30 minutes at 22°C. Worm pieces were rinsed for

10 minutes in 0.5% sodium tungstate in 50% (v/v) ethanol and dehydrated, as described in Section 2.3 but starting with 70% ethanol. Sections were viewed without further staining.

6.2.6 Periodic acid-bismuth subnitrate

Thin sections of adult worms were mounted on nickel grids (see Section 2.3) and stained with alkaline bismuth subnitrate at 22°C following the method of Ainsworth, Ito & Karnovsky (1972).

Grids were floated, section side down, on freshly prepared 1% aqueous periodic acid (Merck, Germany) for 15 minutes. They were washed by repeated dippings (30 to 40) in each of 3 changes of distilled water, followed by 1 minute under a stream of distilled water. Sections were stained by floating them on bismuth subnitrate for 60 minutes (see Ainsworth et al., 1972) and washed as described previously, except that the first rinse was in 0.01N sodium hydroxide.

To control for the aldehyde, carbonyl or other reducing groups already present or introduced by fixation, tissue was fixed in the absence of osmium tetroxide, or treatment of sections with periodic acid was omitted (Lewis & Knight, 1977). Aldehyde groups were blocked with 1M m-aminophenol in glacial acetic acid for 60 minutes at 22°C, before and after oxidation with periodic acid (Lewis & Knight, 1977).

6.2.7 Lectins

Live third-stage larvae, isolated from a mouse 1 hour after infection, and adult worms (see Section 2.2.2) were labelled with the lectins Con A (Sigma Chemical Co., USA) and WGA (Sigma Chemical Co., USA) conjugated to fluorescein isothiocynate (FITC) at 0°C.

Prior to labelling, live worms were incubated in 0.1M glycine in 0.1M Sörensen's phosphate buffer pH 7.2 for 1 hour, to block aldehyde

groups present on the surface of the worms (Schwarz & Koehler, 1979). Following a brief rinse in the same buffer, worms were incubated in the lectins (200 µg per ml of Sörensen's phosphate buffer pH 7.2) for 30 minutes at 0°C, and then washed briefly in the same cold buffer. Control worms were incubated in a mixture of either Con A and 0.1M α-methyl-D-mannoside (Sigma Chemical Co., USA), or WGA and ovomucoid (5 mg per ml; Type III-O, Sigma Chemical Co., USA) for 30 minutes 0°C, and then washed in buffer containing the appropriate sugar.

Worms were immobilized under coverslips by withdrawing the excess moisture, and viewed using a x40 objective lens under epi-illumination in a Zeiss Photomicroscope 1 equipped with a selective FITC filter (BP 485, FT 510, 515-565). Kodak Tri-X pan film (400 ASA) was used.

Each time lectins were used to label worms (see also Chapter 7), spermatozoa from rats were labelled as a positive control because the lectin-binding pattern of these cells is known (Lewin, Wissenberg, Sobel, Marcus & Nebel, 1979; Wright, E. J., 1982). The epididymis was removed from a rat and minced into small pieces in 0.9% sodium chloride. The tissue was incubated for 15 minutes at 37°C to allow the sperm to swim clear of the tissue. The thick suspension of cells was washed twice with 0.9% sodium chloride and then a few drops were placed on clean coverslips and allowed to settle (Wright, 1982). Spermatozoa that attached to the coverslip were fixed in 2.5% paraformaldehyde in 0.1M Sörensen's phosphate buffer pH 7.2, and labelled as described above. Unfixed spermatozoa were also labelled.

6.2.8 Sodium dodecyl sulphate (SDS)

Third-stage and adult *N. dubius* were exposed to 1% SDS (lauryl sulfate; Sigma Chemical Co., USA) for between 1 and 7 days. Adults were

then labelled with cationized ferritin (see Section 6.2.1) and prepared for transmission electron microscopy (see Section 2.3).

Cuticles were isolated from adult worms following the procedure of Cox et al. (1981b). Worms, rinsed 3 times in 0.85% sodium chloride and twice in 0.05M Tris-HCl buffer pH 7.4, were sonicated in 5 ml of the same buffer with glass beads (Sigma Chemical Co., USA) until the red colouration of the worms had disappeared (5 to 10 minutes). Fragments of worm were washed 3 times in the same buffer, and then suspended in 0.125M Tris-HCl buffer pH 6.8, containing 1% SDS, for 3 minutes at 70°C before being left to stand for 1 hour at 22°C. The fragments of cuticle were extracted 4 times and prepared for transmission electron microscopy (see Section 2.3).

6.2.9 β-mercaptoethanol

Adult cuticles, isolated using 1% SDS (see Section 6.2.8), were exposed to 5% β-mercaptoethanol (Merck-Schuchardt, Germany) in 0.125M Tris-HCl buffer pH 6.8, containing 1% SDS for 3 minutes at 70°C and then left for 2 hours at 22°C (Cox et al., 1981b). The extraction procedure was repeated once. The fragments of cuticle were rinsed several times in 0.125M Tris-HCl buffer pH 6.8, followed by 3 rinses in 0.1M Sörensen's phosphate buffer pH 7.2, and fixed overnight in 3% glutaradehyde in 0.1M phosphate buffer pH 7.2, before being prepared for transmission electron microscopy (see Section 2.3).

6.2.10 Elastase (EC 3.4.21.36)

Cuticles isolated from adult worms (see Section 6.2.8) were labelled with cationized ferritin (see Section 6.2.1) and subsequently exposed to elastase from porcine pancreas (Type I, Sigma Chemical Co., USA) following the method of Cox et al. (1981b). Cuticles were exposed

to elastase (0.5 mg per ml) in 0.05M sodium acetate buffer pH 8.8, for 1, 4 or 24 hours at 37°C. Fragments from the first 2 treatments were relabelled with cationized ferritin (see Section 6.2.1) and all were prepared for transmission electron microscopy (see Section 2.3).

Ensheathed third-stage larvae were exposed to elastase (0.5 mg per ml) in 0.05M sodium acetate buffer, pH 8.8 for 4 hours at 37°C. Control worms were incubated in either the buffer, or distilled water for 4 hours, at either 37°C or 22°C. Because larvae were still active after treatment with elastase, they were not prepared for transmission electron microscopy. Instead the effect of elastase on the permeability of the cuticle to glutaraldehyde was assessed as follows. Larvae exposed to elastase were treated with 3% glutaraldehyde in 0.1M Sörensen's phosphate buffer pH 7.2, at 22°C. The length of time these worms survived in glutaraldehyde was compared to a control group, not pre-treated with elastase.

6.2.11 Hyaluronidase (EC 3.2.1.35)

The effect of hyaluronidase (bovine testes, 350 NF units per mg; Sigma Chemical Co., USA) on the cuticle of artificially exsheathed third-stage larvae of N. dubius was tested (see Section 3.2). Exsheathed larvae were exposed to 1% hyaluronidase in 0.05M sodium acetate buffer pH 6.0, containing 0.85% sodium chloride for either 1 or 15 hours at 22°C, or 1 hour at 37°C. Control worms were treated in the same way except that the enzyme was omitted.

Activity of the enzyme was tested on the cumulus cells surrounding the ova of mice, before and after the experiment. The ova dispersed within 2 minutes of contacting the enzyme in both cases. Ova treated with buffer, on the other hand, did not disperse.

Because larvae were still active after treatment with hyaluronidase, any effect the enzyme might have had on the permeability of the cuticle to glutaraldehyde was tested (see Section 6.2.10).

6.2.12 Sodium hypochlorite

Adults and infective larvae of N. dubius were exposed to 1% sodium hypochlorite ("Milton"; Richardson-Merrell Pty. Ltd., Australia) at 22°C, and observed hourly.

6.2.13 Organic solvents

Adult N. dubius were exposed to 4M guanidine hydrochloride (guanidine-HCl; Sigma Chemical Co., USA) in 0.05M sodium acetate buffer pH 5.6, for between 1 and 24 hours at 22°C. They were subsequently labelled with cationized ferritin (see Section 6.2.1) and prepared for transmission electron microscopy (see Section 2.3).

When third-stage larvae were treated with 4M guanidine-HCl in 0.05M sodium acetate buffer pH 5.7, for 1 hour at 22°C, the supernatant was removed and tested for uronic acid following the method of Bitter & Muir (1962). To 5 ml of sulphuric acid which had been chilled to 4°C, 1 ml of supernatant was added, and the tube was shaken. The mixture was heated for 10 minutes in a boiling water bath and cooled, before 0.2 ml of carbazole reagent was added. The optical density was read at 530 nm.

Adult worms, in 0.2 ml of 0.85% sodium chloride, were added to 1.8 ml of water containing 2 gm of phenol (BDH Chemical Co., UK). The test tube was maintained at between 68 to 70°C for 30 minutes with intermittant agitation (Westphal & Jann, 1965). Worms were removed from the mixture while it was still warm because phenol precipitates at low temperatures. The worms were then prepared for electron microscopy (see Section 2.3).

Adults were also placed in a mixture of 2-chloroethanol (Aldrich Chemical Co., USA) and sulphuric acid (12 parts of 2-chloroethanol: 16 parts of 73% sulphuric acid) or formic acid (BDH Chemicals Ltd., UK) for between 1 and 24 hours at 22°C. Tissue surviving this treatment was labelled with cationized ferritin (see Section 6.2.1) and prepared for electron microscopy (see Section 2.3).

Infective-stage larvae were exposed to butanol (Ajax Chemicals, Australia), diethyl ether (Ajax Chemicals, Australia) and chloroform: methanol (3:1; Ajax Chemicals, Australia) at 22°C for 1 hour.

6.2.14 Sodium periodate

Infective-stage larvae were exposed to 2% aqueous sodium periodate (Merck, Germany) for 1 hour at 22°C. Because larvae were active after this time, any effect sodium periodate might have had on the permeability of the cuticle to glutaraldehyde was tested as described in Section 6.2.10.

6.2.15 Association between second-stage larvae and E. COLI

First- and second-stage worms were cultured as described in Section 2.2.1. Pre-moults were removed from the culture medium, washed 5 times in Krebs-Ringer salt solution buffered with 0.1M phosphate buffer pH 6.0, and incubated overnight in the same buffer at 26°C so that the second moult took place in the absence of E. coli. These larvae were exposed to dead E. coli, live E. coli or human erythrocytes in Krebs-Ringer salt solution buffered with 0.1M phosphate buffer pH 6.0, for 2 hours at 22°C. One hundred larvae from each treatment were then checked for adhering cells.

Other second-stage worms were pre-treated either, with neuraminidase (10 units per ml in sodium acetate buffer pH 5.5; Type V,

Sigma Chemical CO., USA; Gress & Lumsden, 1976a), or with 0.1M mannose (α-methyl-D-mannoside; Sigma Chemical CO., USA) for 2 hours at 22°C, and then exposed to dead E. coli suspended in Krebs-Ringer salt solution buffered with 0.1M phosphate buffer pH 6.0 for a further 2 hours. Worms were washed several times in the same buffer, and one hundred from each treatment were checked for adhering cells.

A hundred larvae allowed to develop in the culture medium containing killed E. coli were also checked for adhering cells.

6.3 RESULTS

6.3.1 Cationized ferritin

Cationized ferritin bound to the surface of the adult cuticle, as well as to the first-, second- and fourth-stage cuticles (Figs. 6.1, 6.2 and Figs 6.5 to 6.9). Labelling of the third-stage cuticle was attempted twice but no cationized ferritin was detected on the surface of any of the 10 larvae that were examined (Fig. 6.3). Erythrocytes, which were labelled in the same square watch glass as the larvae as a control, agglutinated in the presence of cationized ferritin. And thin sections of the red blood cells revealed dense particles of cationized ferritin on the surface of these cells (Fig. 6.4).

The distribution of cationized ferritin adhering to the surface of N. dubius varied between stages. First- and second-stage cuticles were usually coated with a single layer of dense particles (Figs 6.1 and 6.2), but occasionally groups of molecules were observed.

In contrast, the distribution of cationized ferritin on the surface of the fourth-stage and adult cuticle was irregular (Figs 6.5 to 6.9). Less cationized ferritin bound to the fourth-stage cuticle

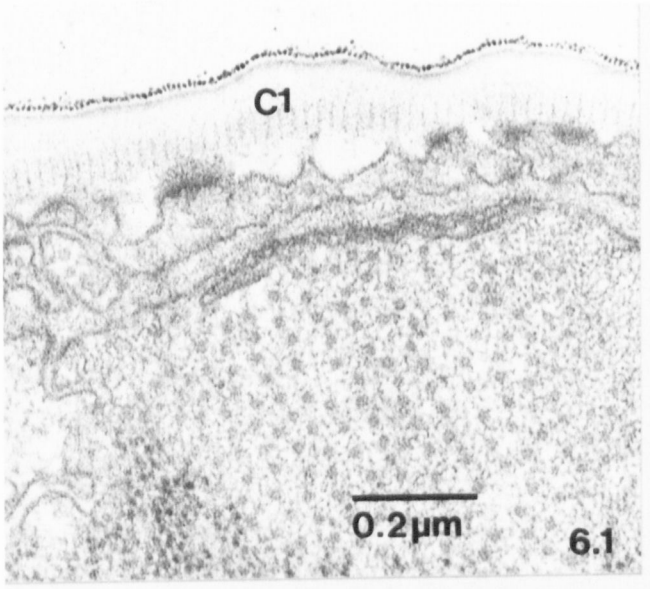
Fig. 6.1: The surface of a first-stage larva of Nematospiroides dubius labelled with cationized ferritin. For the most part, a single row of molecules of cationized ferritin is present along the surface of the cuticle (Cl).

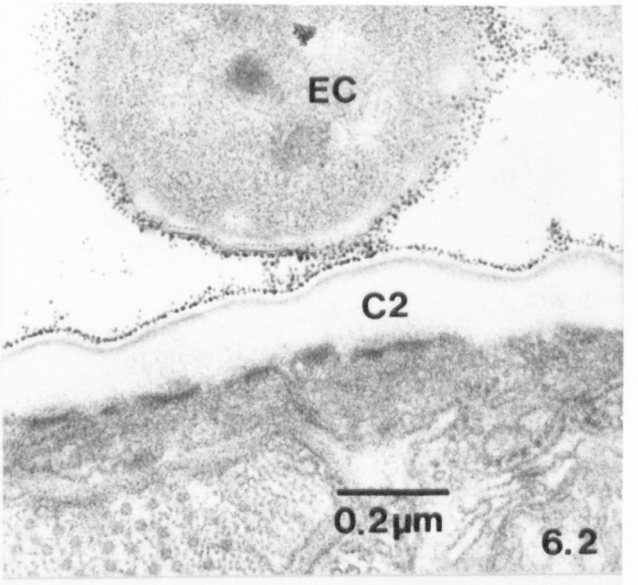
Fig. 6.2: Cationized ferritin bound to the surface of a second-stage larva of Nematospiroides dubius. Note that Escherichia coli (EC) from the culture medium also adhere to the cuticle (C2).

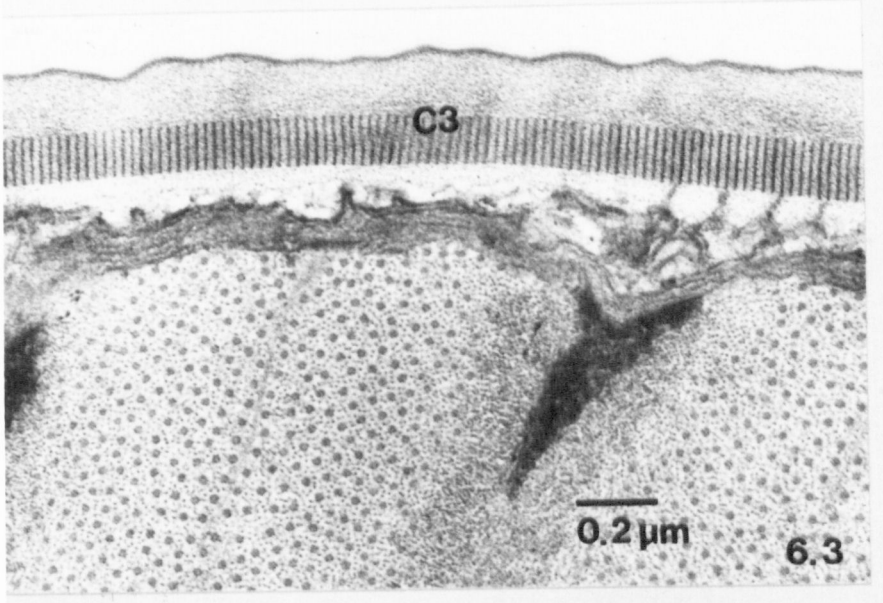
Fig. 6.3: When artificially exsheathed larvae of Nematospiroides dubius were incubated in cationized ferritin, no electron-dense particles were detected on the surface of third-stage cuticle (C3)

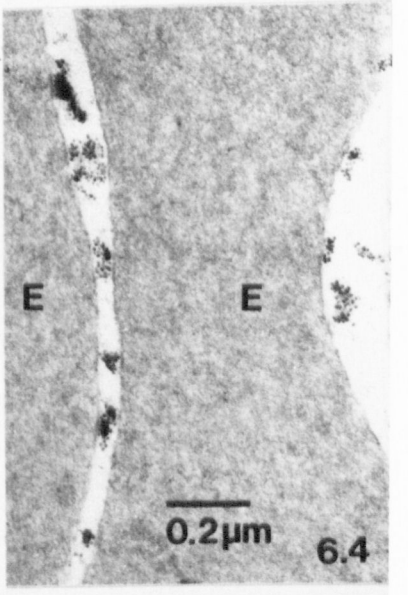
Fig. 6.4: Erythrocytes (E), labelled with cationized ferritin as a control for infective-stage larvae, agglutinated in the presence of cationized ferritin. Cationized ferritin adhered to the surface of these cells (compare with Fig. 6.3).

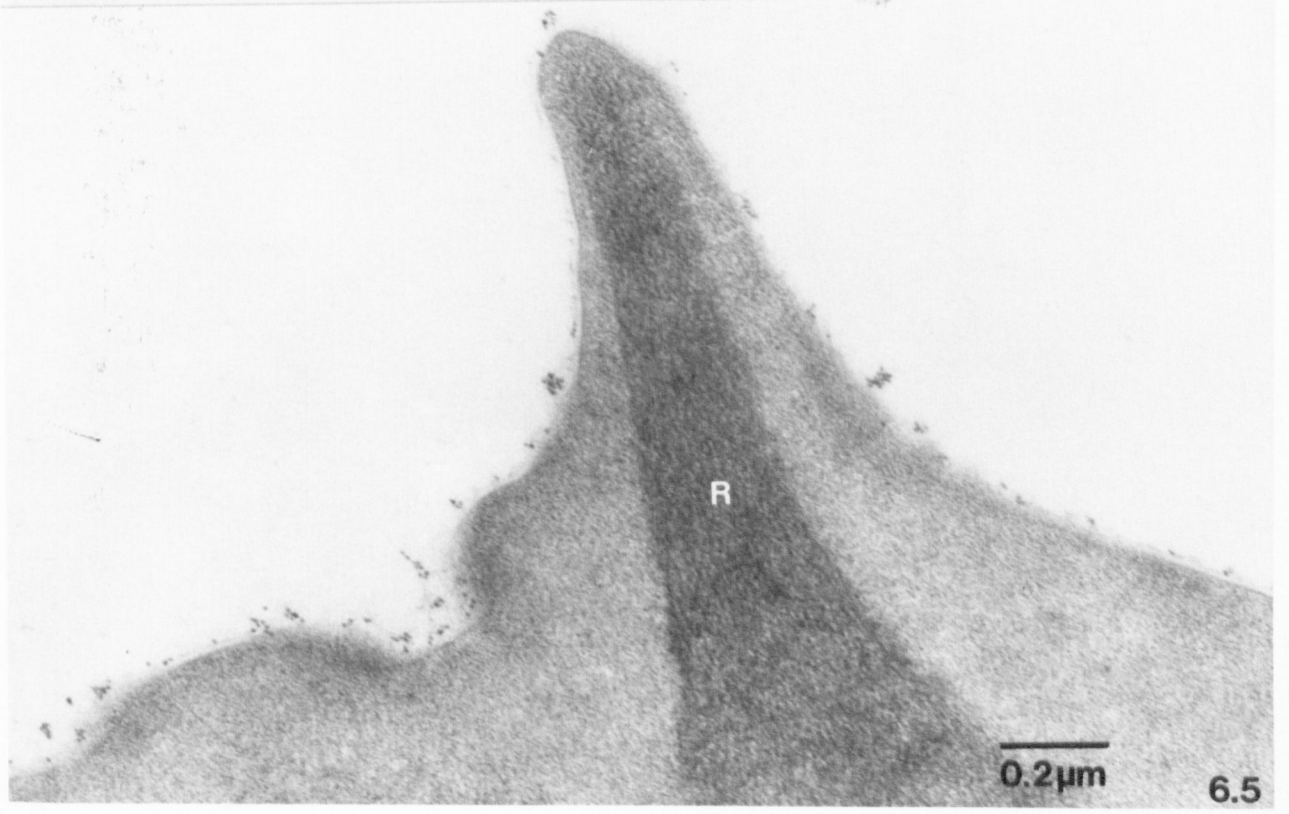
Fig. 6.5: A few molecules of cationized ferritin adhered to the surface of the fourth-stage larva of Nematospiroides dubius. Note that some electron-dense particles adhered to the tip of the cuticular ridge (R).


C1
0.2µm
6.1


EC
C2
0.2µm
6.2


C3
0.2µm
6.3


E
E
0.2µm
6.4


R
0.2µm
6.5

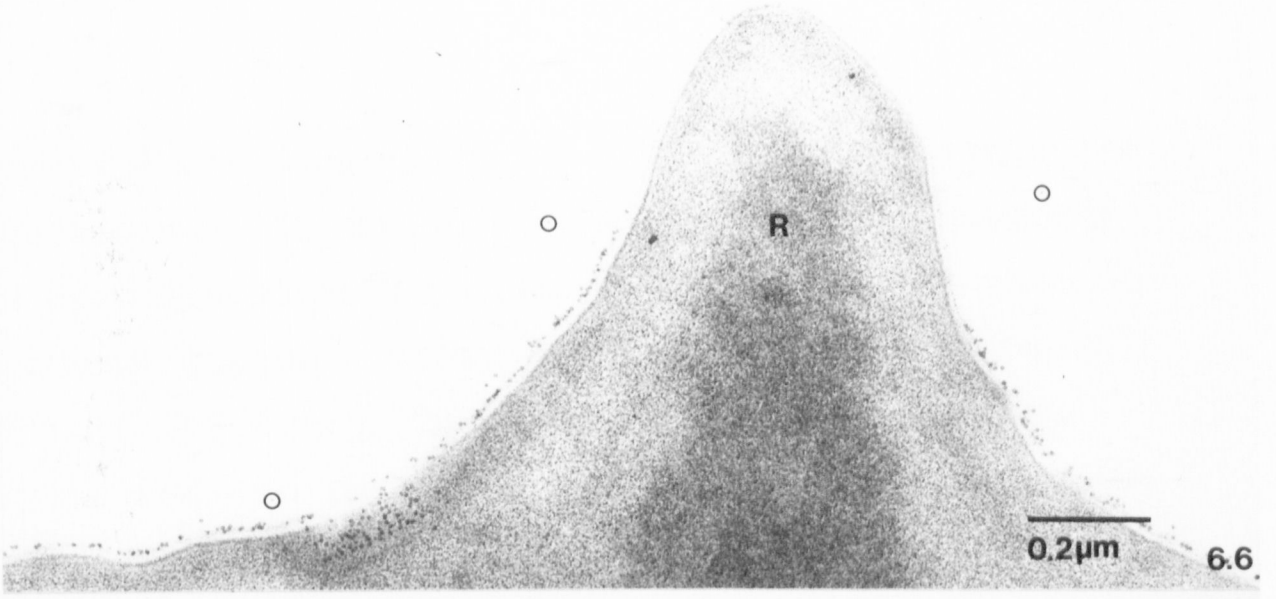
Figs 6.6 to 6.9: Micrographs depicting the surface of adult Nematospiroides dubius which were labelled with cationized ferritin.

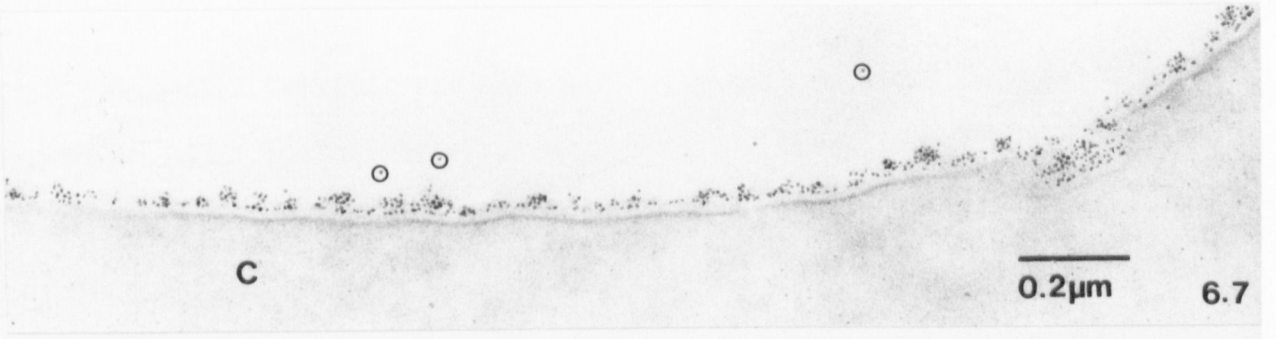
Fig. 6.6: The tip of the cuticular ridge (R) is free of cationized ferritin but the label is present on the rest of the cuticle surface. Several electron-dense particles are present above the surface of the worm (circled). The section was lightly stained with uranyl acetate and lead citrate.

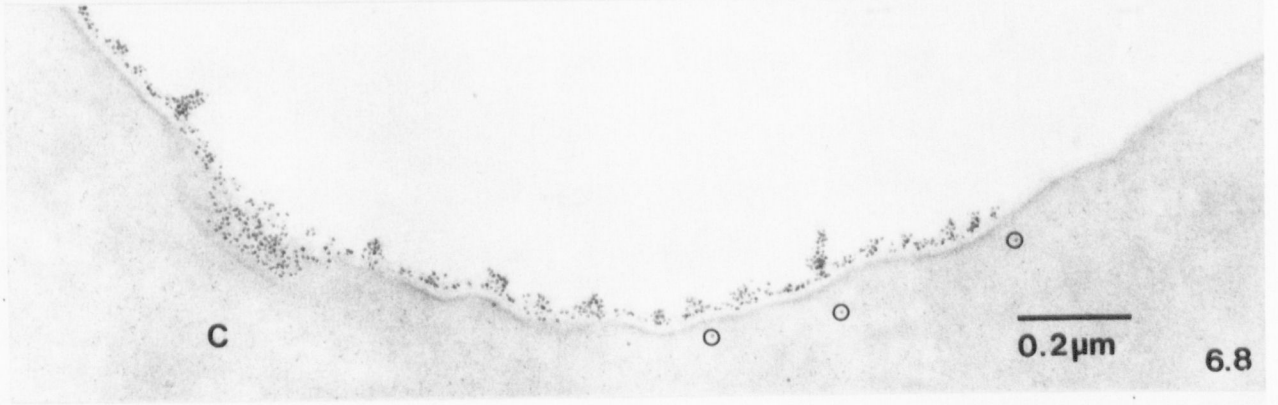
Fig. 6.7. An unstained section in which electron-dense particles (circled) are present in the resin above the surface of the cuticle (C).

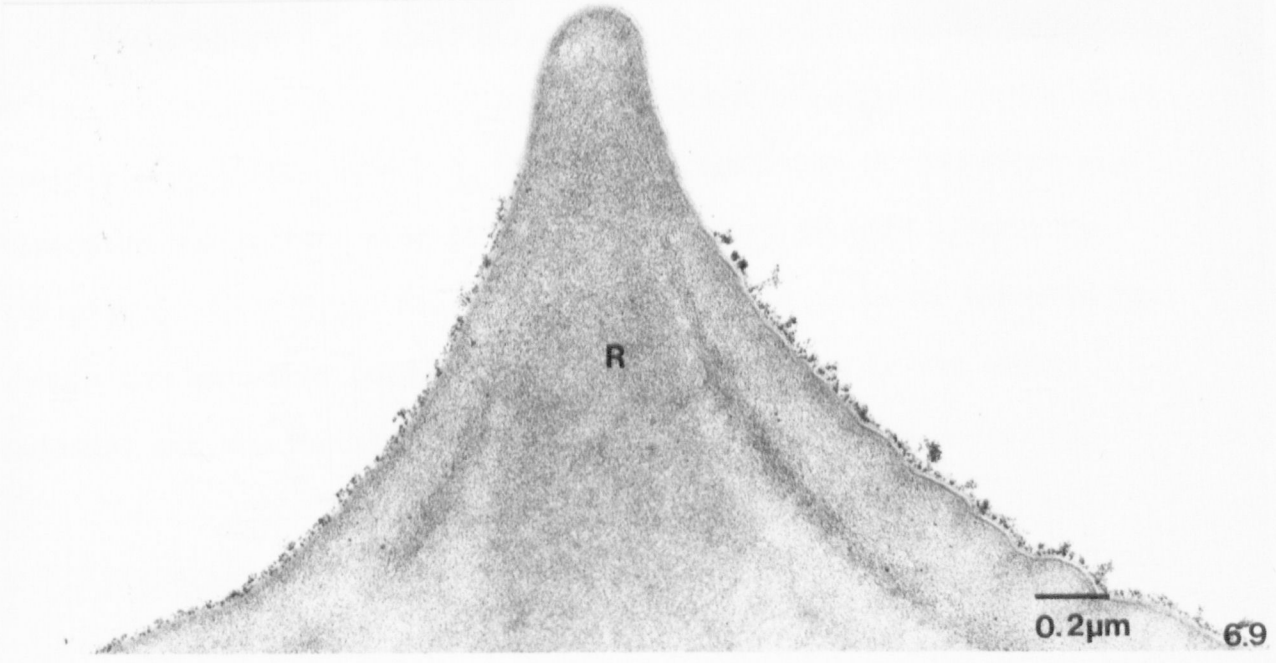
Fig. 6.8: An unstained section in which electron-dense particles are present (circled) within the cuticle (C).

Fig. 6.9: Cationized ferritin present on the surface of an adult worm isolated from a host 8 months after infection. Note that the tips of the cuticular ridge (R) did not label.


R
0.2μm
6.6


C
0.2μm
6.7


C
0.2μm
6.8


R
0.2μm
6.9

compared with the adult cuticle, and its distribution was more patchy. Moreover, while cationized ferritin was never detected on the tips of the longitudinal ridges of the adult cuticle, a few dense particles were observed in this region of the fourth-stage cuticle (see also Figs 3.13 and 3.14). The number and distribution of molecules of cationized ferritin adhering to the surface of the adult cuticle did not appear to alter as the worms aged within the host (compare Figs 6.6 to 6.8 with Fig. 6.9).

Cationized ferritin was detected along most of the length of adult male worms using microprobe analysis. Only the bursa were consistently found to be free of ferritin (see Appendix 1). However, because counts made over a given area lacked consistency, the method was deemed unsatisfactory for quantifying ferritin on nematodes (see Appendix 1).

Cationized ferritin did not appear to penetrate the cuticle of N. dubius (Figs 6.1 to 6.3 and Figs 6.5 to 6.9), although dense particles were sometimes visible within unstained sections of the cuticle of adult worms (Fig. 6.8). But because similar particles were sometimes seen in the resin surrounding the worm, they were assumed to be an artefact of section cutting (Fig. 6.7).

Pre-treatment of adult worms with neuraminidase had no detectable effect on the extent to which cationized ferritin adhered to the adult cuticle (Figs 6.10 and 6.11). The same preparation of neuraminidase disrupted the surface coat of eggs of N. dubius in this laboratory (Wright, E. J., 1982). Poly-L-lysine, on the other hand, appeared to reduce the amount of cationized ferritin that bound to the adult cuticle, but the results were not consistent (Figs 6.12 and 6.13).

6.3.2 Ruthenium red

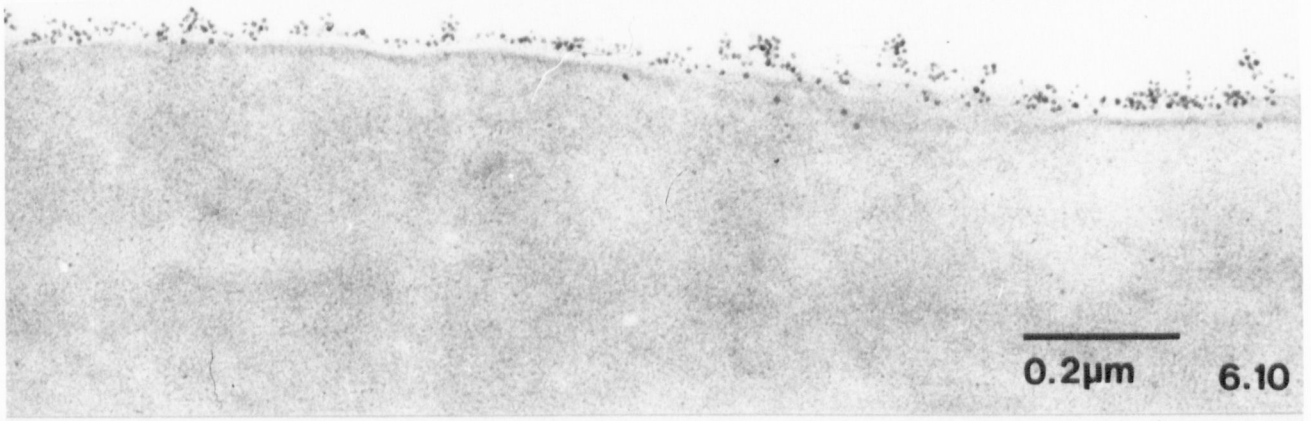
When adult worms were stained with ruthenium red, a dense "fuzzy"

Fig. 6.10: A stained section through the cuticle of an adult Nematospiroides dubius, pre-treated with neuraminidase and labelled with cationized ferritin (compare with Figs 6.6 to 6.8).

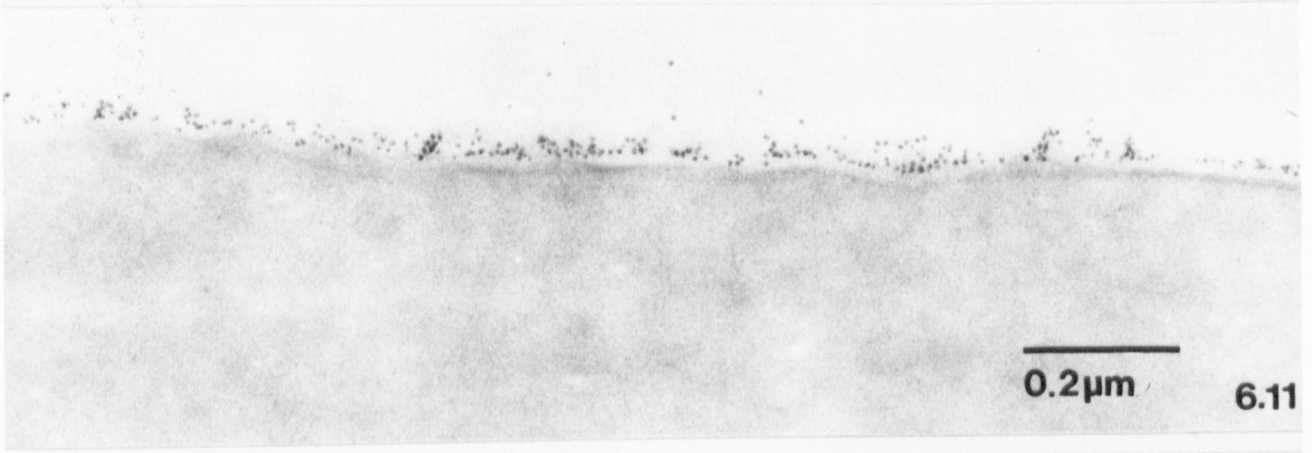
Fig. 6.11: An unstained section through the cuticle of an adult Nematospiroides dubius, pre-treated with neuraminidase and labelled with cationized ferritin (compare with Figs 6.6 to 6.8).

Fig. 6.12: Pre-treating adult Nematospiroides dubius with poly-L-lysine usually reduced the amount of cationized ferritin that adhered to the surface (compare with Figs 6.6 to 6.8 and Figs 6.10 and 6.11). The section was lightly stained with uranyl acetate and lead citrate.

Fig. 6.13: Pre-treating adult Nematospiroides dubius with pol-L-lysine, sometimes resulted in clumps of cationized ferritin adhering to the surface (compare this unstained section with Fig. 6.12).

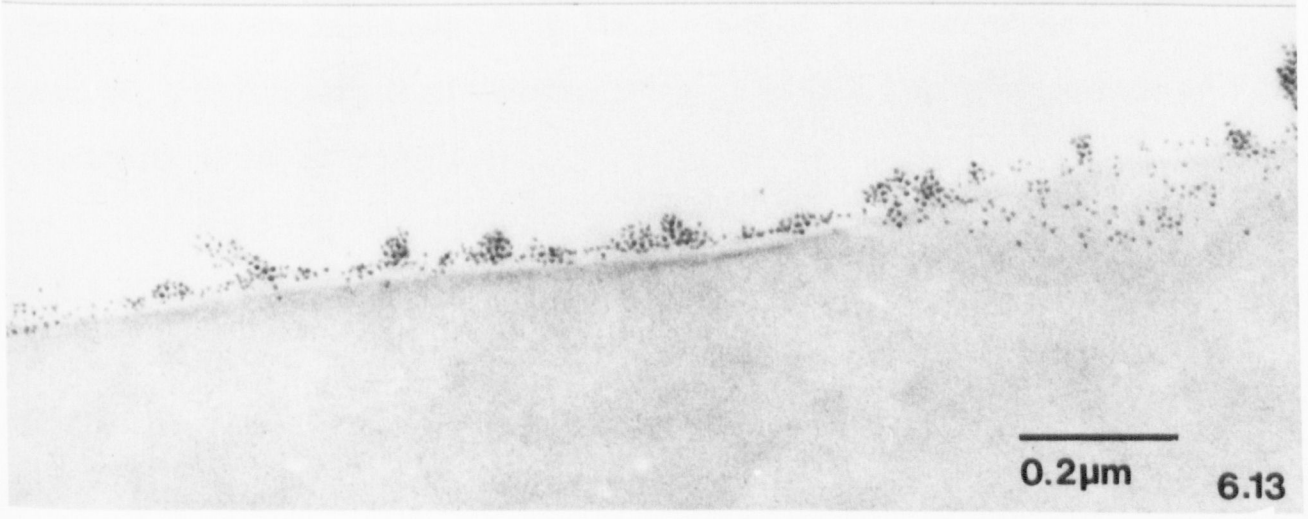


6.10



6.11

6.12



6.13

precipitate measuring between 20 and 55 nm in thickness was visible over the surface (Figs 6.14 and 6.15). Sometimes layering could be detected within this surface coat (Figs 6.14 to 6.17). Unlike cationized ferritin, ruthenium red also stained the tips of the cuticular ridges, but here the coat was only between 9 and 16 nm thick (Fig. 6.15). Ruthenium red enhanced the trilaminate appearance of the epicuticle by giving the inner and outer lines equal density. In control worms, the inner line of the epicuticle was more dense than the outer one, and the surface coat could hardly be detected (Fig. 6.21).

Pre-treatment with neuraminidase (see Section 6.3.1 for note on activity of enzyme) had no detectable effect on the way in which ruthenium red stained the surface of the adult cuticle (Figs 6.16 and 6.17).

The staining properties of the surface of infective-stage larvae differed markedly from those of the adult cuticle. Ruthenium red stained the surface of the second-stage sheath and the third-stage cuticle (compare Figs 6.18 and 6.19 with Fig. 6.25). The surface of the sheath appeared as a trilaminate structure bounded by a short electron-dense surface coat between 6 and 10 nm thick (Figs 6.18). No precipitate was visible on the surface of the third-stage cuticle, but the outer layers of epicuticle appeared more dense than those of control worms (Figs 6.18, 6.19 and 6.25). An amorphous precipitate of ruthenium red was sometimes associated with the surface of the third-stage cuticle, particularly in ensheathed worms, and with the inner surface of the sheath (Figs 6.18 and 6.19).

6.3.3 Alcian blue-lanthanum nitrate

The surface of adult worms also stained when alcian blue was added to the aldehyde fixative at a neutral pH (compare Figs 6.20 and 6.21).

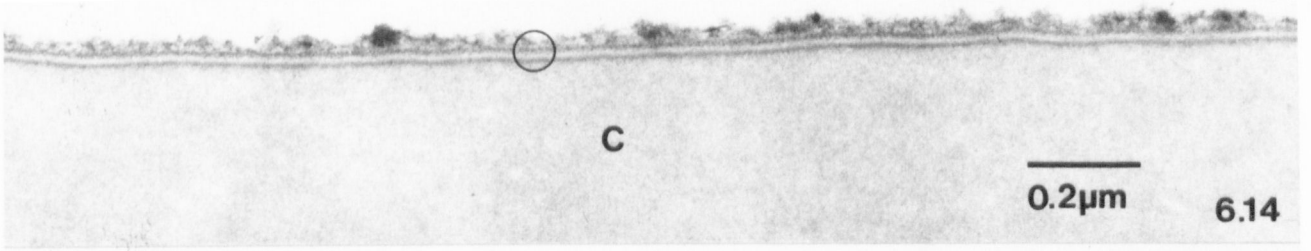
Figs 6.14 to 6.17: Micrographs depicting the surface of adult Nematospiroides dubius following staining with ruthenium red. The sections were not counter-stained.

Fig. 6.14: The surface coat and trilaminate structure bounding the cuticle (C) are intensely stained by the dye. In some regions (circled) the surface coat appears layered.

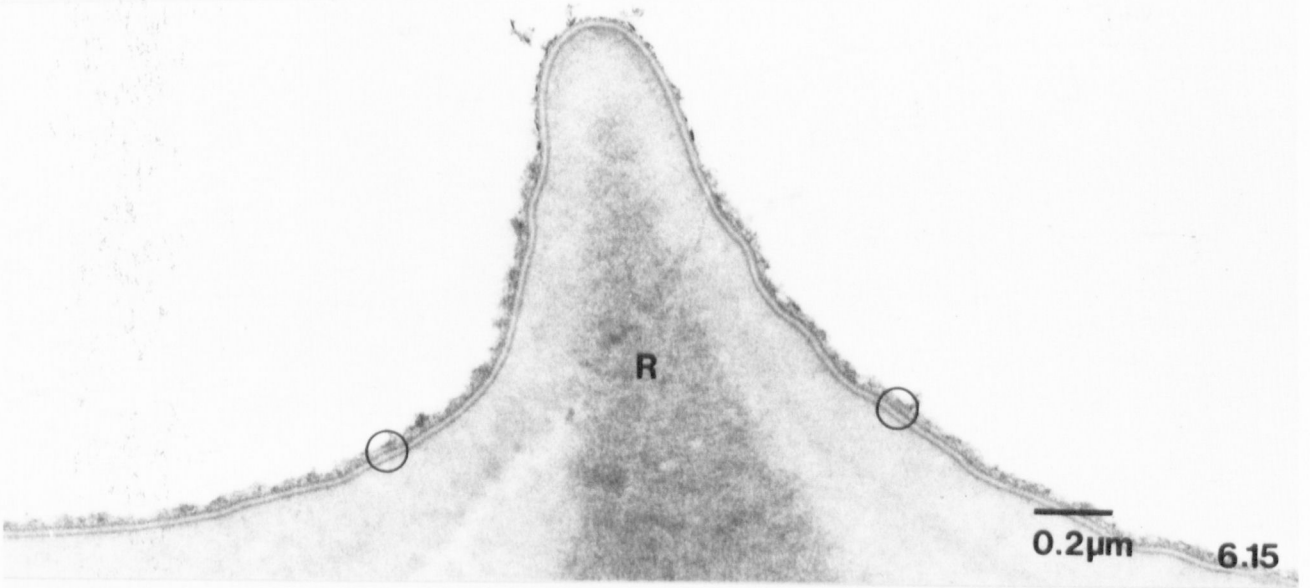
Fig. 6.15: The tip of a cuticular ridge (R) exhibits a dense precipitate when stained with ruthenium red (compare with Fig. 6.6). In some regions (circled) the surface coat appears layered.

Fig. 6.16: The surface coat of adult worms pre-treated with neuraminidase and then stained with ruthenium red did not differ from untreated worms (compare with Fig. 6.14). Note the layered appearance of the surface coat in some regions (circled).

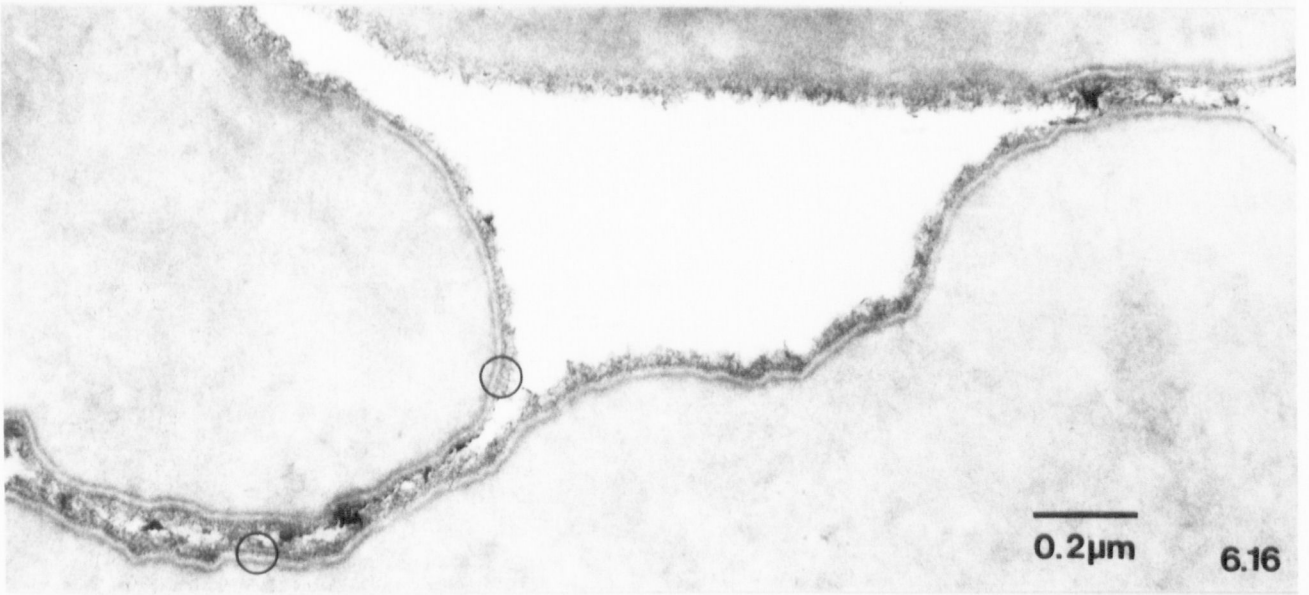
Fig. 6.17: Pre-treatment of adult worms with neuraminidase did not affect the pattern of staining obtained using ruthenium red. In some regions the surface coat appears layered (circled - compare with Figs 6.15 and 6.16).



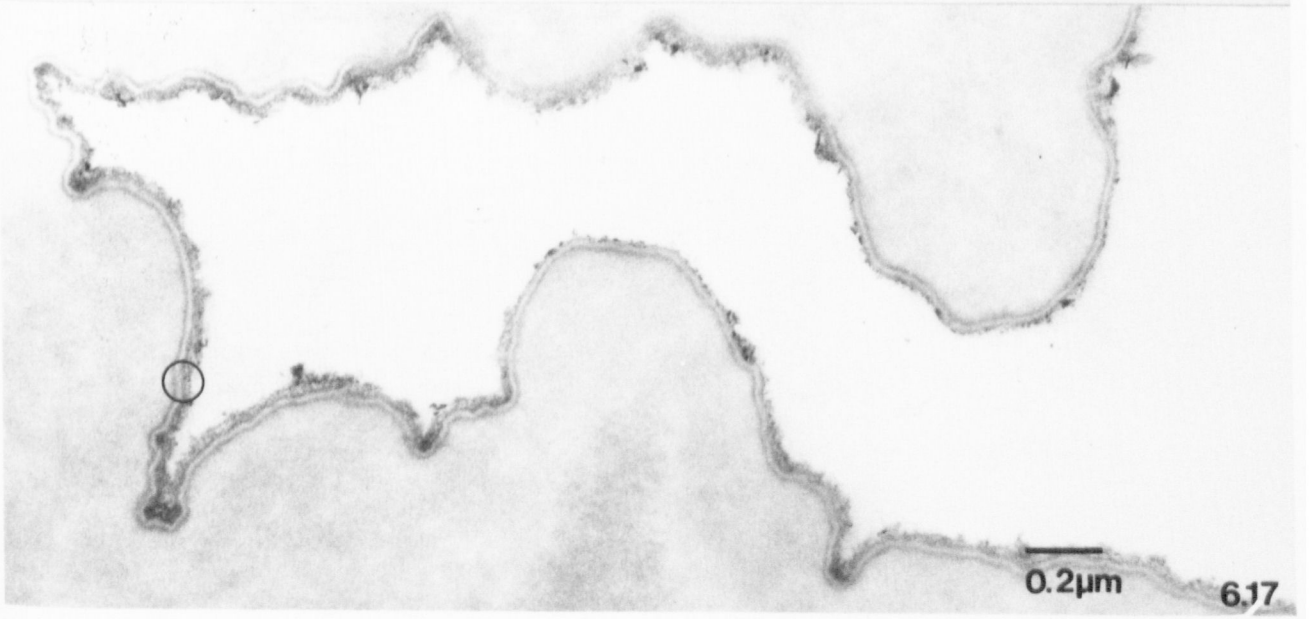
6.14



6.15



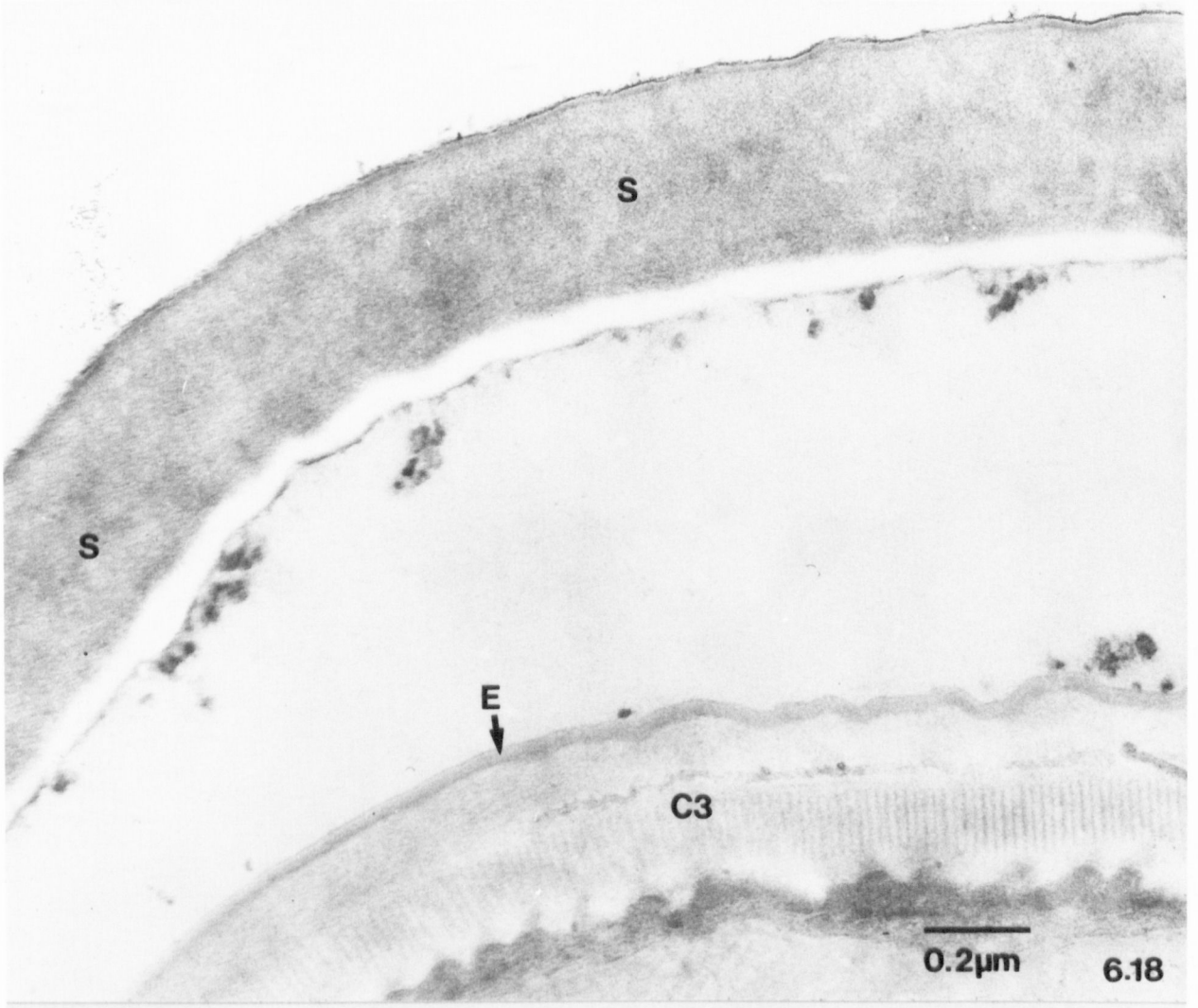
6.16



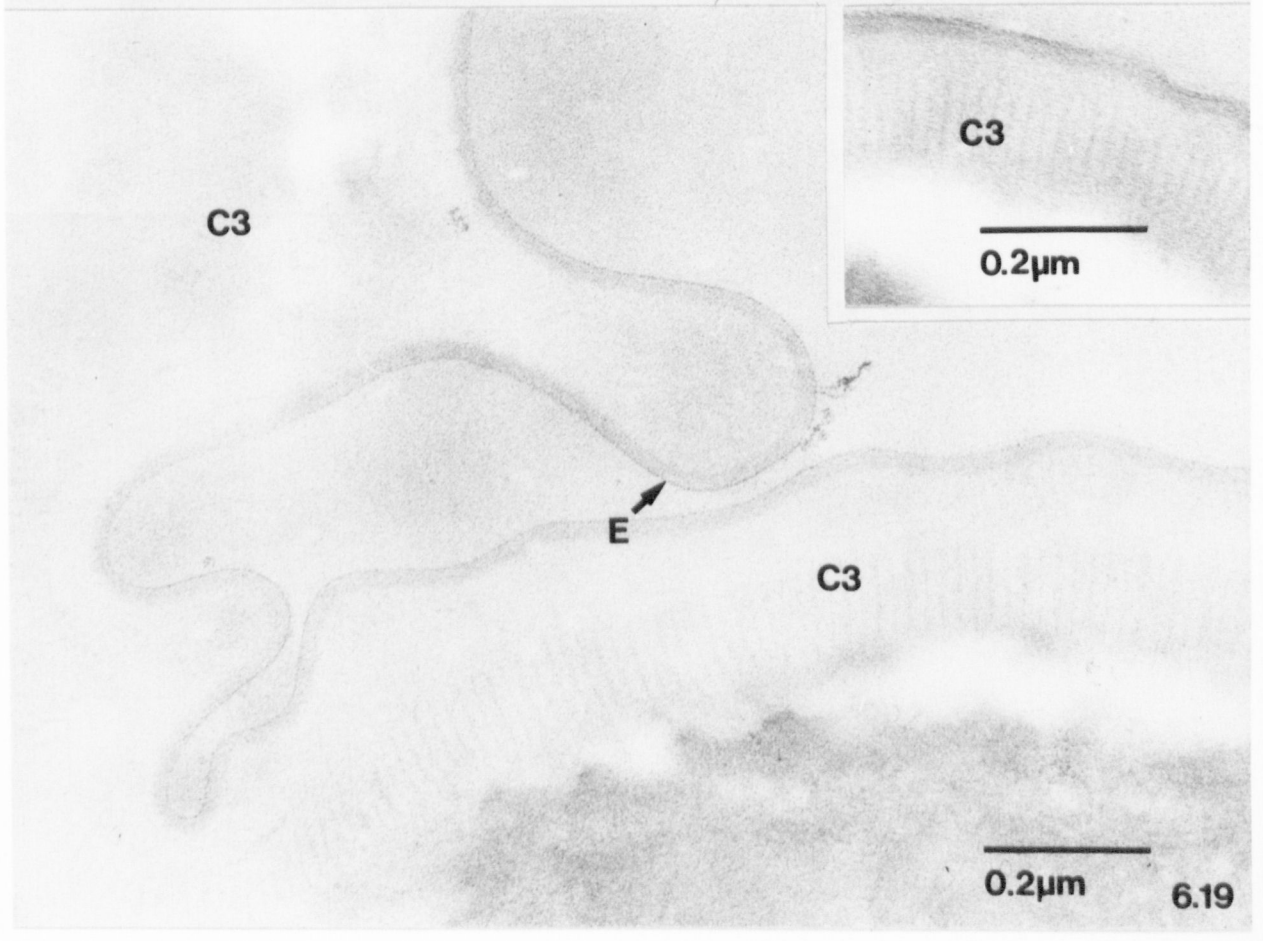
6.17

Fig. 6.18: An ensheathed larva of Nematospiroides dubius stained with ruthenium red. An electron-dense precipitate is present on the surface of the second-stage sheath (S). Patches of ruthenium red are associated with the inner boundary of the sheath, and with the surface of the third-stage cuticle (C3). The outermost layers of the epicuticle (E) of the C3 appear electron-dense (compare with Fig. 6.25). The section was not counter-stained.

Fig. 6.19: When exsheathed infective larvae of Nematospiroides dubius were stained with ruthenium red, the outer region of the epicuticle (E) of the third-stage cuticle (C3) appeared electron-dense (also see inset) but no surface coat was visible (compare with Fig. 6.25). The section was not counter-stained.



6.18

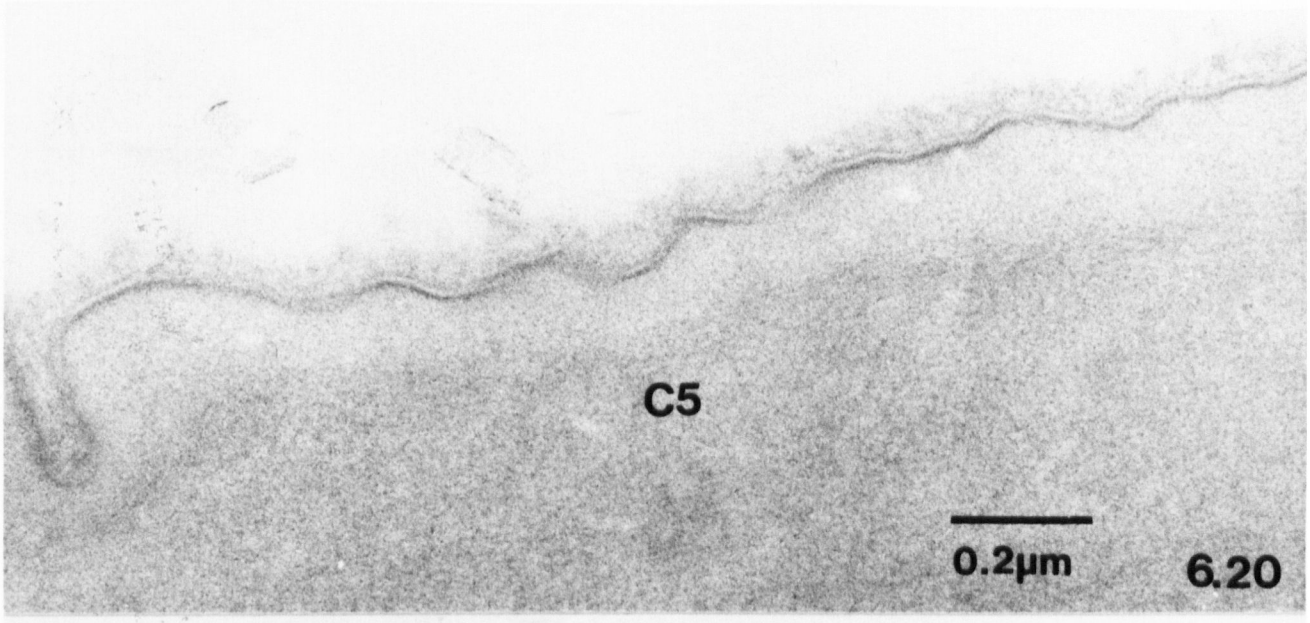


6.19

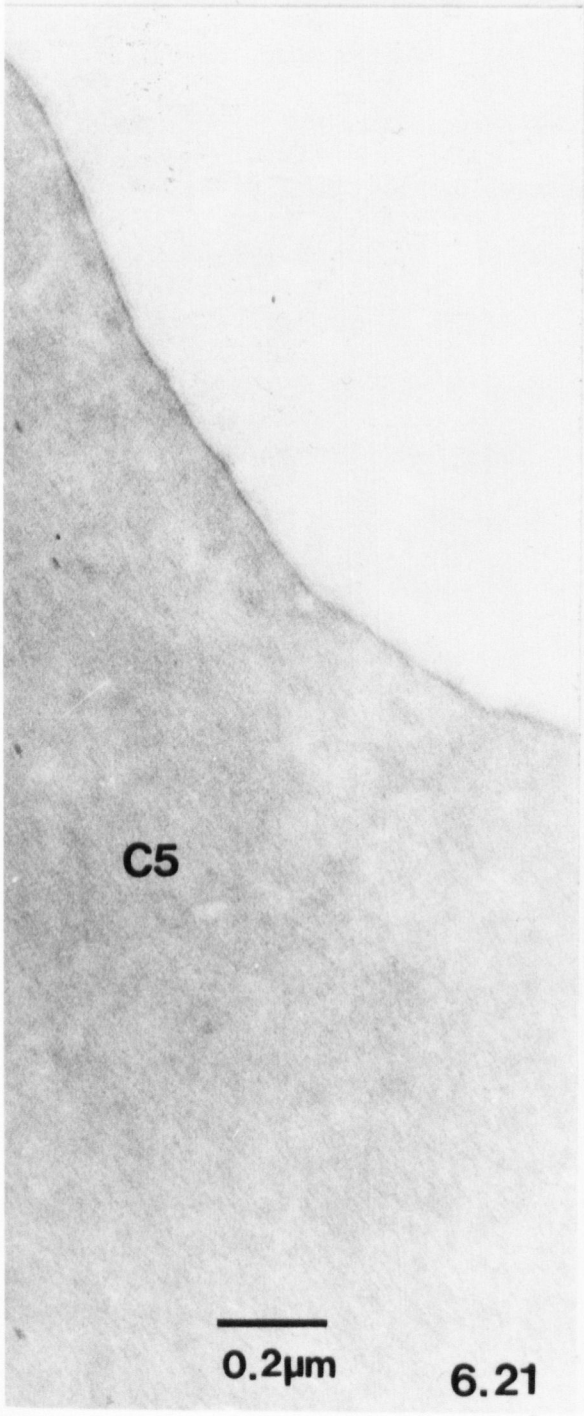
Fig. 6.20: An unstained section through the adult cuticle (C5) of Nematospiroides dubius, fixed in glutaraldehyde containing alcian blue at neutral pH, and post-fixed in osmium tetroxide containing lanthanum nitrate. Note that the inner line of the epicuticle is more dense than the outer one, and the surface coat has a filamentous appearance (compare with Figs 6.14 and 6.15).

Fig. 6.21: An unstained section through the cuticle (C5) of an adult Nematospiroides dubius fixed in gluraldehyde and osmium tetroxide (compare with Figs 6.14 to 6.17 and Fig. 6.20). Note that the inner line of the epicuticle is more dense than the outer one, and the surface coat is difficult to detect.

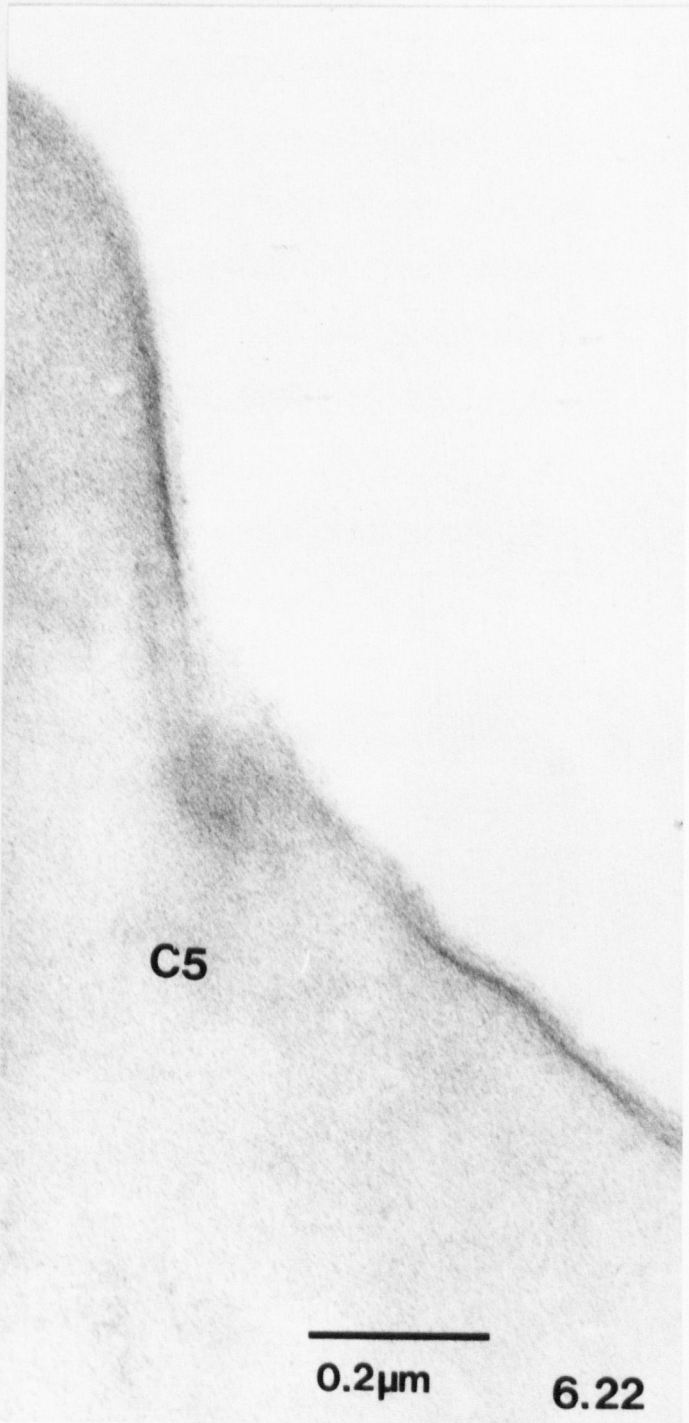
Fig 6.22: The cuticle (C5) of an adult Nematospiroides dubius fixed in glutaraldehyde followed by osmium tetroxide. The contrast of the section was enhanced by staining with lead citrate for 3 minutes (compare with Figs 6.23 and 6.24).



6.20



6.21



6.22

The "tram-line" appearance of the epicuticle was highlighted but the inner line was still more electron-dense than the outer one (compare Figs 6.20, 6.14 and 6.6). The surface coat was well-preserved using this method, and measured between 50 and 80 nm in thickness. It appeared longer and more filamentous than when stained with ruthenium red (compare Figs 6.20 and 6.14).

6.3.4 Tannic acid

Tannic acid improved the contrast of the adult cuticle. The fibre layers of the basal zone were particularly clear when worms were stained with tannic acid following fixation (Fig. 6.24). The surface coat and trilaminate structure of the epicuticle were highlighted when tannic acid was used either as a stain, or as a fixative but the appearance of the coat depended on the method used (Figs 6.23). Where tannic acid was employed as a mordant for lead staining, the surface coat appeared short (9 to 20 nm) and felt-like (Fig. 6.24). On the other hand, when tannic acid was used in the fixative, the surface coat appeared longer (20 to 40 nm) and was composed of fine filaments (Fig. 6.23).

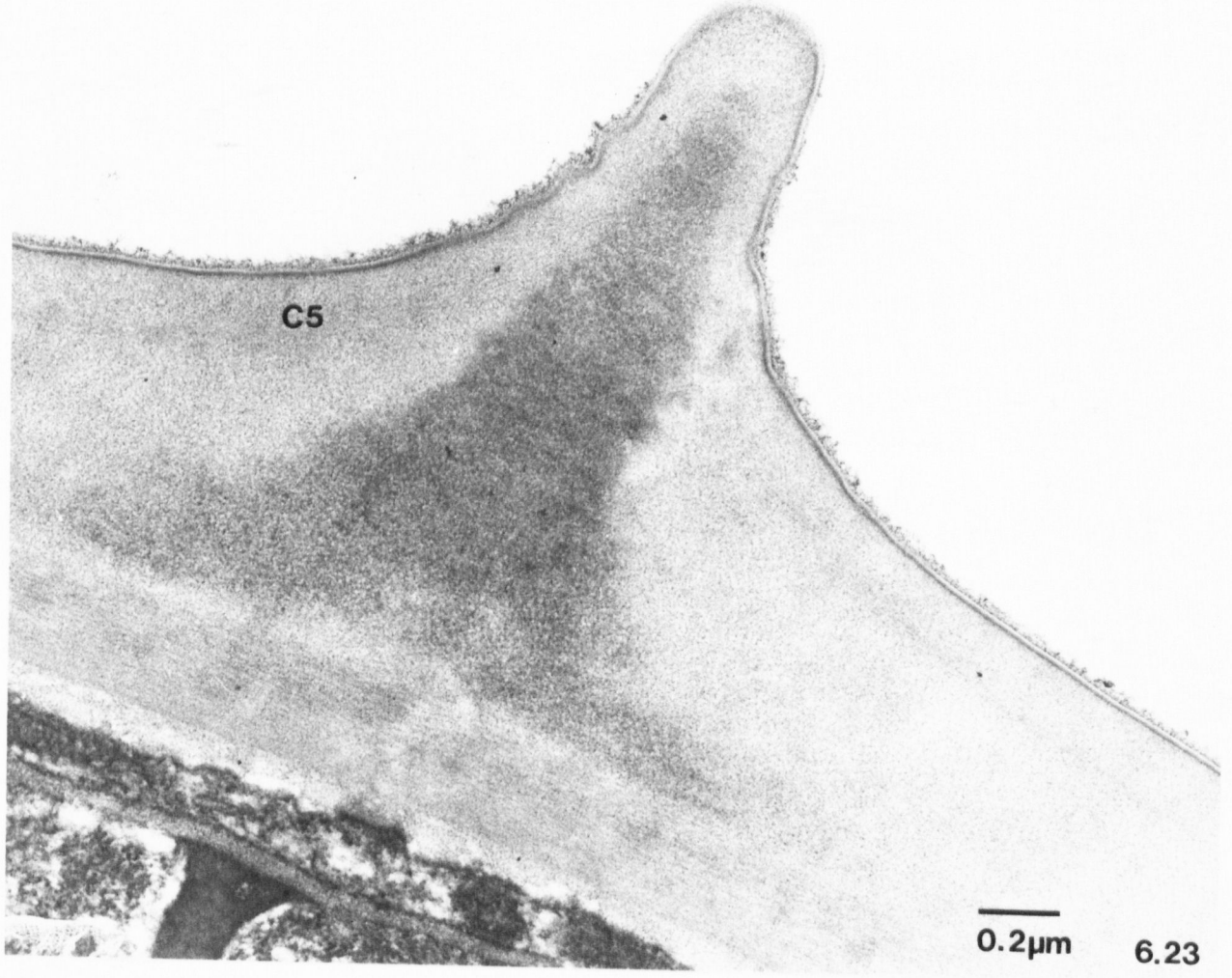
The outermost line of the epicuticle of the third-stage larvae appeared electron-dense which ever method was used (compare Fig. 6.26 with Figs 6.27 and 6.28). A dense precipitate was also sometimes present on the surface of the third-stage cuticle. Where ensheathed larvae were fixed in tannic acid followed by staining in uranyl acetate, the sheath appeared black.

6.3.5 Cuprolinic blue

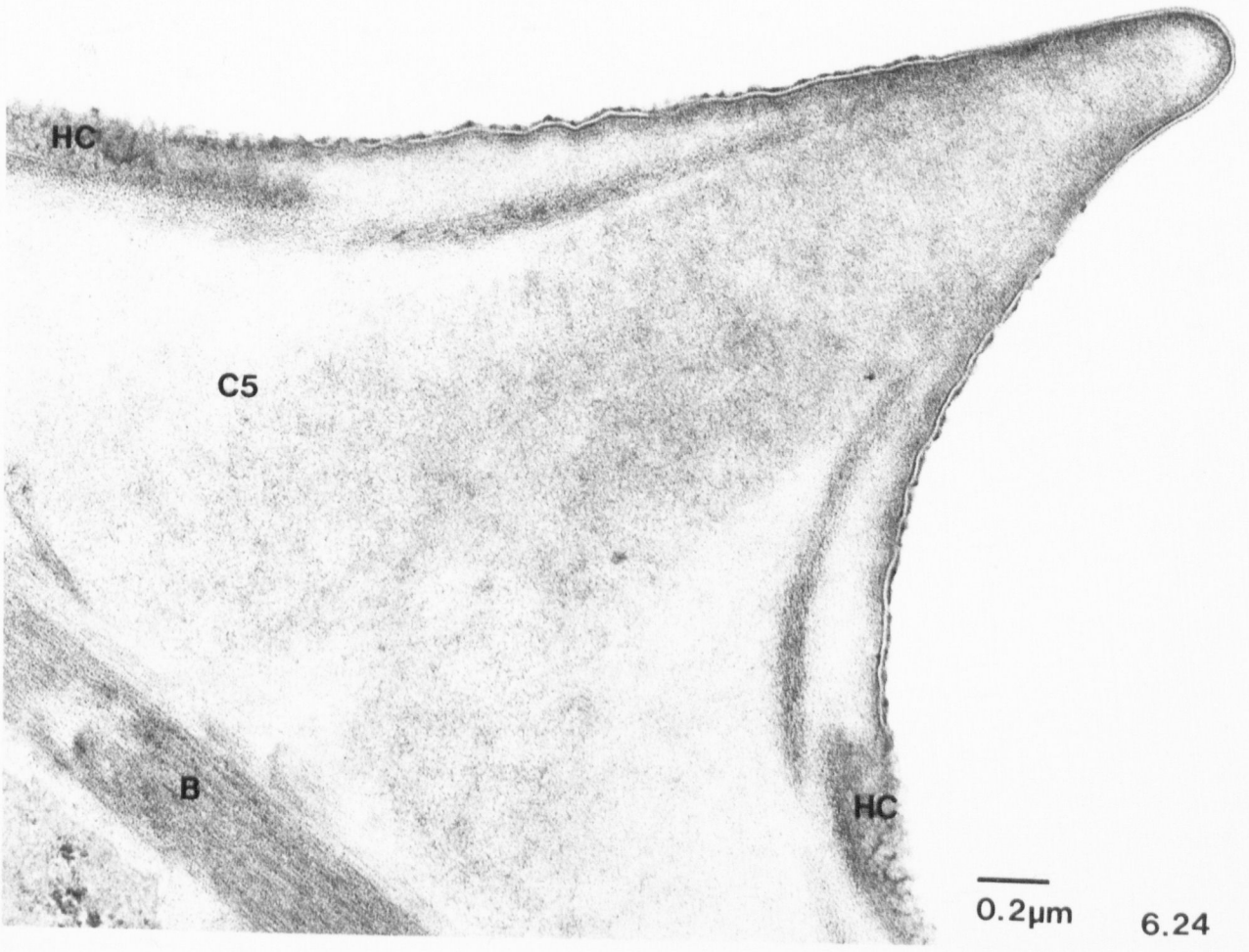
Unlike the stains already mentioned, cuprolinic blue did not highlight the surface coat of the adult cuticle, although the layers making up the epicuticle were stained (Figs 6.29 and 6.30). The central

Fig. 6.23: The cuticle (C5) of an adult Nematospiroides dubius fixed in glutaraldehyde containing tannic acid and stained for 6 hours in uranyl acetate. The section was counter-stained with lead citrate for 3 minutes. The contrast of the trilaminate structure bounding the cuticle has been sharply increased (compare with Fig. 6.22) and the surface coat appears filamentous.

Fig. 6.24: The cuticle (C5) of an adult Nematospiroides dubius stained with tannic acid following fixation. The section was counter-stained for 3 minutes with lead citrate. The trilaminate surface structure has been highlighted (compare with Fig. 6.22) and the surface coat appears "felt-like" (compare with Fig. 6.23). The fibres of the basal zone (B) were well preserved by the method and a "honeycomb pattern" (HC) can be seen within the cuticle.



6.23



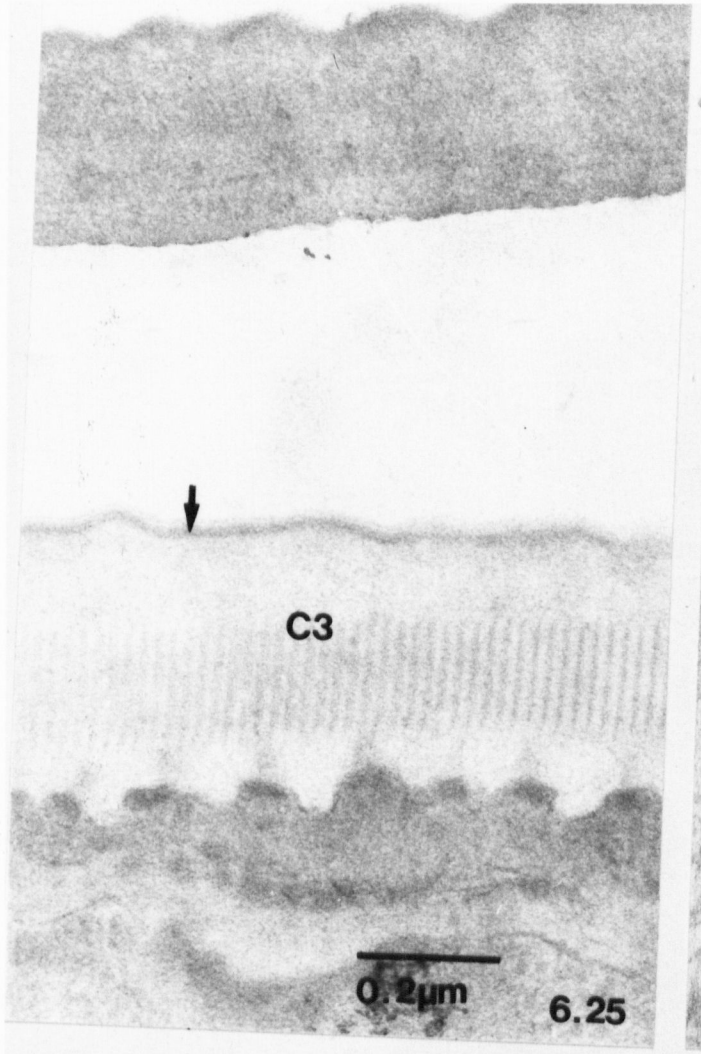
6.24

Fig. 6.25: An unstained section through a third-stage larva of Nematospiroides dubius fixed in glutaraldehyde and post-fixed in osmium tetroxide. The inner layers (→) of the epicuticle of the third-stage (C3) cuticle are more dense than the outer ones.

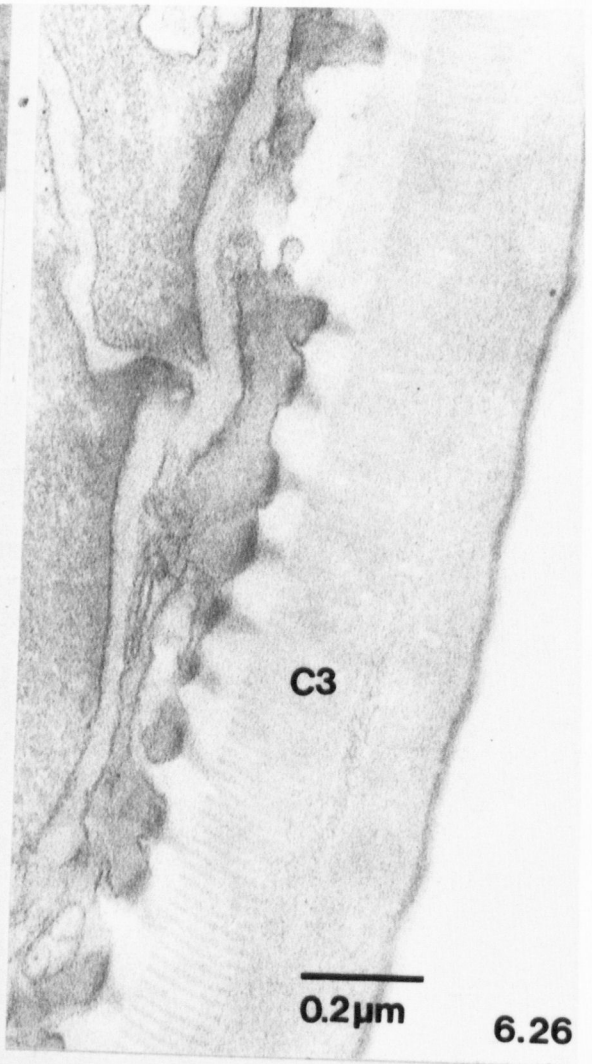
Fig. 6.26: The cuticle (C3) of an infective larva of Nematospiroides dubius fixed in glutaraldehyde followed by osmium tetroxide and stained with lead citrate for 3 minutes (compare with Fig. 6.27).

Fig. 6.27: The outer epicuticle of the third-stage cuticle (C3) of an exsheathed larva of Nematospiroides dubius stained with tannic acid appears electron-dense, and a dense precipitate is present on the surface. The section was counter-stained with lead citrate for 3 minutes (compare with Fig. 6.26).

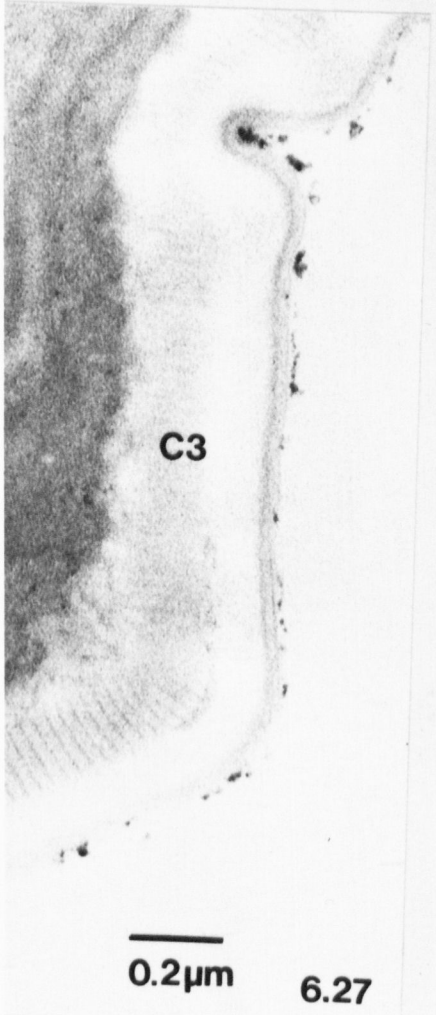
Fig. 6.28: The outer epicuticle of an infective larva of Nematospiroides dubius appears dense following fixation in tannic acid. The sheath (S) is black and a dense precipitate is associated with the surface of the third-stage cuticle (C3).



6.25



6.26



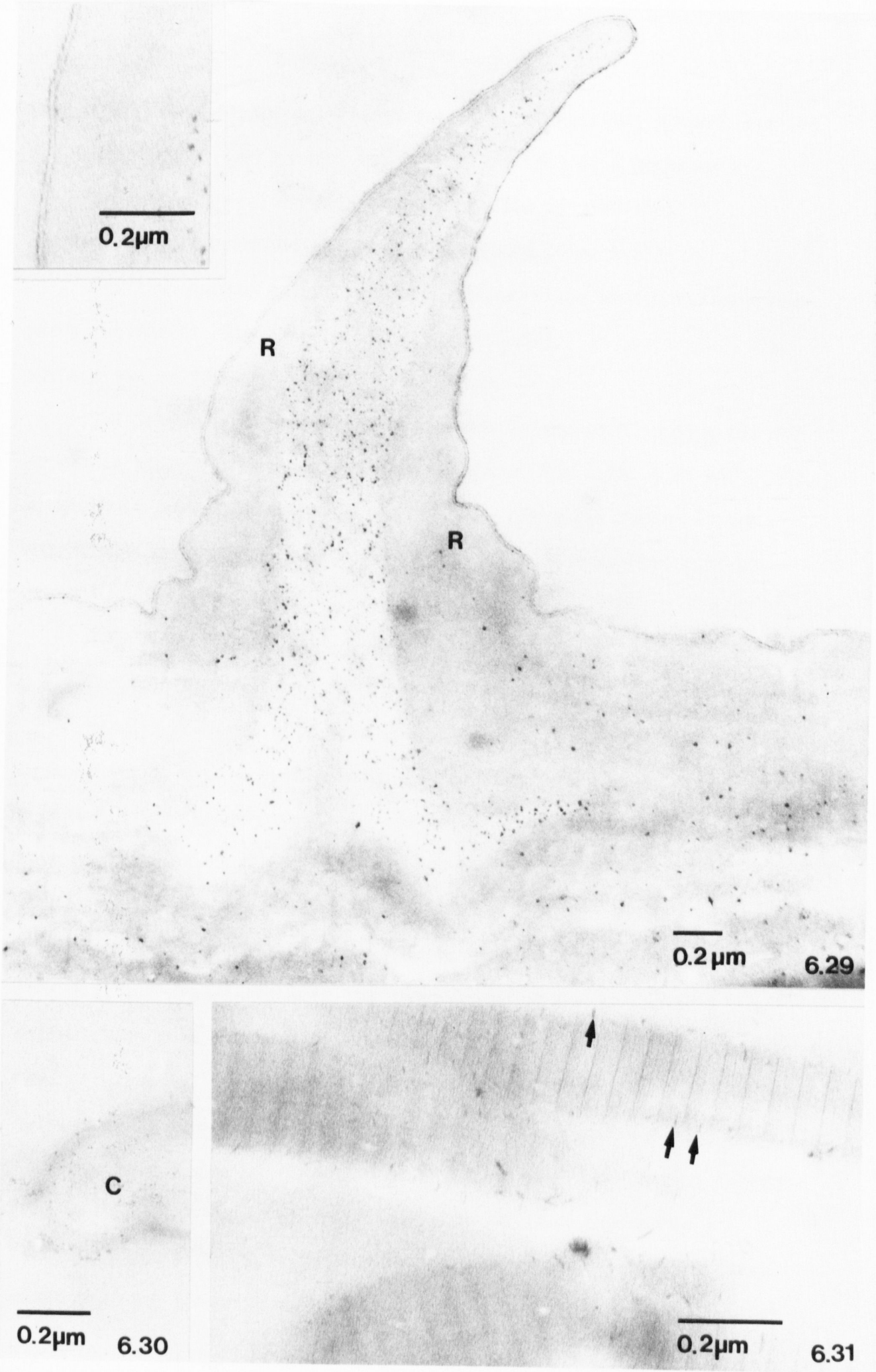
6.27

6.28

Fig. 6.29: The bar within the cuticular ridge (R) of the adult cuticle of Nematospiroides dubius is marked by an accummulation of dense particles following staining with cuprolinic blue. The trilaminate epicuticle also stained with the dye but no surface coat is visible (see inset). The section was not counter-stained.

Fig. 6.30: The cuticle (C) of an adult Nematospiroides dubius worm fixed in glutaraldehyde without osmication as a control for staining with cuprolinic blue.

Fig. 6.31: Transverse section through mouse tail tendons stained with cuprolinic blue. The dense parallel lines represent hoops of proteoglycan. Where the fibrils are cut on the diameter, filaments of proteoglycan are seen in cross-section (→).


0.2µm
R
R
0.2 µm
6.29
C
0.2µm
6.30
0.2µm
6.31

portion of the longitudinal ridges seemed to selectively accummulate the dye (Fig. 6.29). And tendon, taken from the tail of a mouse as a positive control, stained with cuprolinic blue to give the characteristic banded pattern for proteoglycan (Fig. 6.31).

The trilaminate structure of the second-stage sheath was enhanced using cuprolinic blue (Figs 6.32, 6.34 and 6.33). In particular, the surface was coated with a dense precipitate.

The layered epicuticle of third-stage larvae of N. dubius did not appear to stain with cuprolinic blue (compare Figs 6.32, 6.33 and 6.36), although in some micrographs the density of the outer region of the epicuticle appeared to be enhanced (Fig. 6.33). No surface coat was present.

6.3.6 Periodic acid-bismuth subnitrate

Results obtained with periodic acid-bismuth subnitrate were disappointing, even though sections were stained on several occasions. A fine electron dense precipitate was associated with the epicuticle when sections of adult worms, which had been oxidized with periodic acid were stained with bismuth subnitrate (Fig. 6.37). In some cases, the precipitate appeared to be associated with the surface coat, but in others it was associated with the inner layer of the trilaminate epicuticle. When sections from worms, which had not been fixed in osmium tetroxide, were oxidized and stained with bismuth subnitrate, the epicuticle appeared electron-dense and the surface coat was more dense than the inner layer of the epicuticle (Fig. 6.38). In control sections that had not been oxidized (Fig. 6.39), or had been exposed to a blocking agent (Figs 6.40 and 6.41), the surface coat was either less dense than the inner layer of the epicuticle, or the two were of equal density.

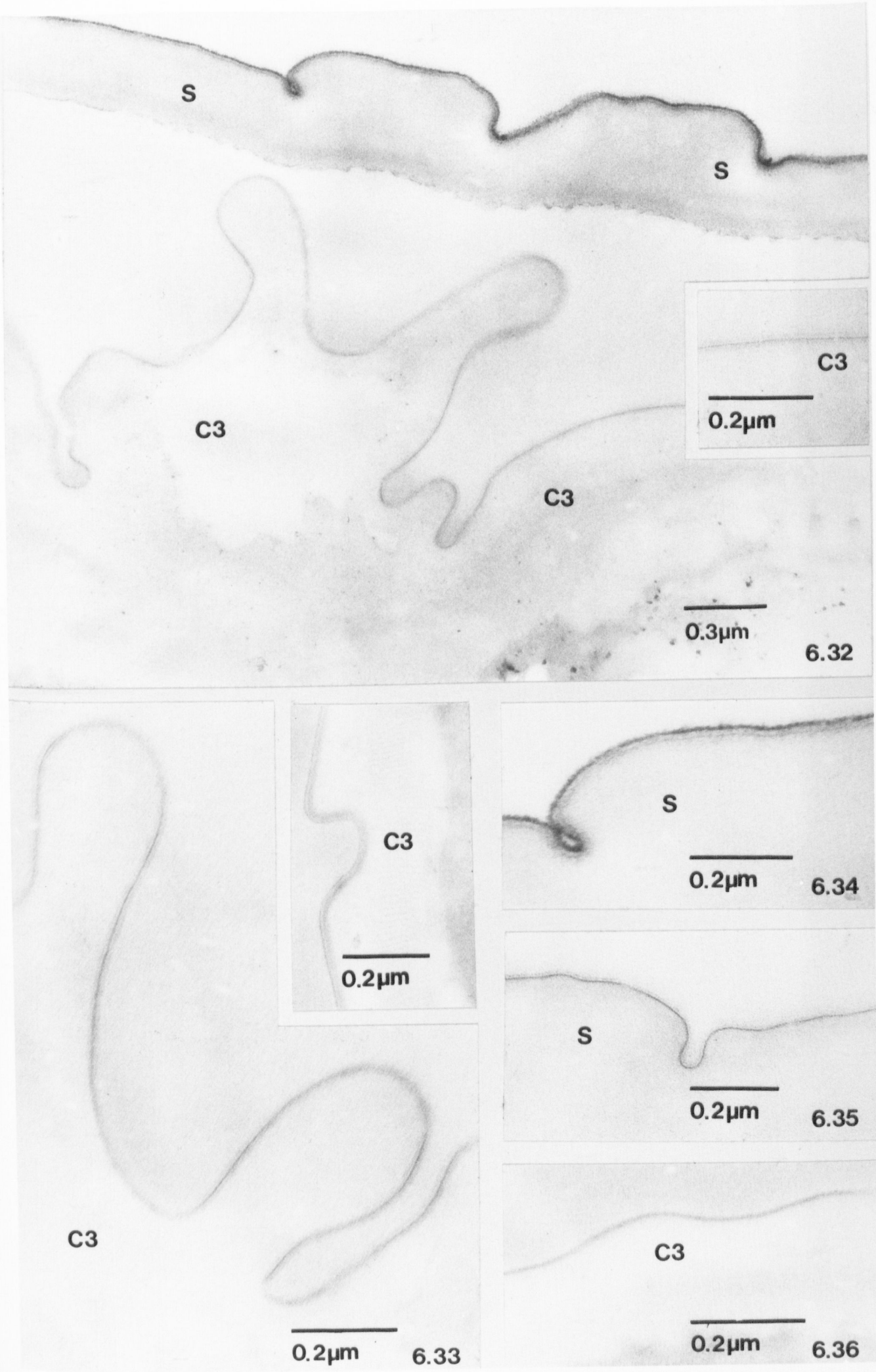
Fig. 6.32: An infective larva of Nematospiroides dubius that was fixed in glutaraldehyde containing cuprolinic blue. The epicuticle of the sheath (S) is clearly visible and an electron-dense precipitate is present on its surface (compare also with Figs 6.34 and 6.35). The outer region of the third-stage cuticle (C3) beneath the sheath is unstained (see inset).

Fig. 6.33: The surface of the cuticle (C3) of a third-stage larva of Nematospiroides dubius, artificially exsheathed and stained with cuprolinic blue (compare with Fig. 6.36). No surface coat can be seen.

Fig. 6.34: The sheath (S) of an infective larva of Nematospiroides dubius stained with cuprolinic blue (compare with Figs. 6.32 and 6.35).

Fig. 6.35: The second-stage sheath (S) stained only with sodium tungstate as a control for cuprolinic blue (compare with Figs 6.32 to 6.34). The inner line of the epicuticle is dense but there is no surface coat.

Fig. 6.36: The cuticle (C3) of a third-stage larva stained only with sodium tungstate as a control for cuprolinic blue (compare with Fig. 6.33). The surface has little electron density.


S
S
C3
C3
0.2µm
C3
0.3µm
6.32
C3
0.2µm
S
0.2µm
6.34
S
0.2µm
6.35
C3
C3
0.2µm
6.33
0.2µm
6.36

Figs 6.37 to 6.41: The adult cuticle of Nematospiroides dubius stained with bismuth subnitrate.

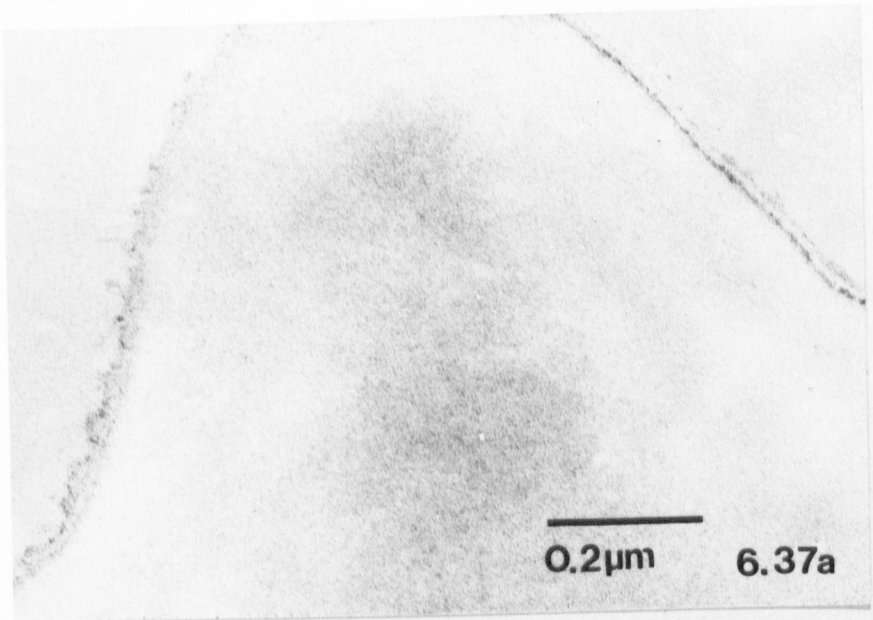
Fig. 6.37 (a - c): A fine precipitate is associated with the epicuticle in sections oxidized with periodic acid and stained with bismuth subnitrate.

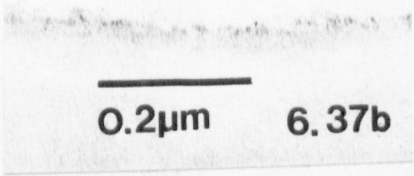
Fig. 6.38: The surface of worms, not exposed to osmium tetroxide, appeared electron-dense when oxidized with periodic acid and stained witn bismuth subnitrate.

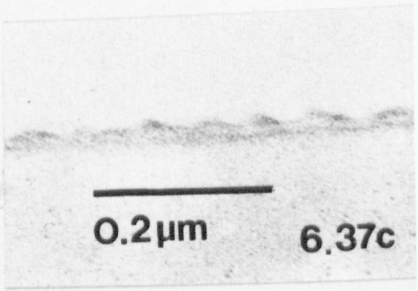
Fig. 6.39: Where bismuth subnitrate was used without oxidation, the surface coat was less dense than the inner line of the epicuticle.

Fig. 6.40: The surface coat appears slightly less dense than and the inner layer of the epicuticle when sections were treated with 1M m-aminophenol in glacial acetic acid after oxidation.

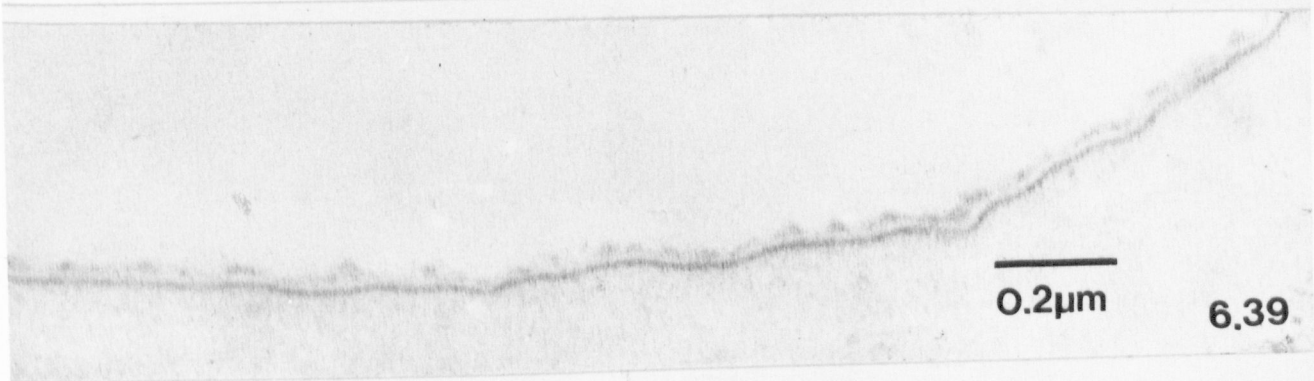
Fig. 6.41: In this micrograph of a section, treated with 1M m-aminophenol in glacial acetic acid before oxidation, the cuticle shows some electron-density but the inner and outer layers of the epicuticle have a similar density.


0.2µm
6.37a

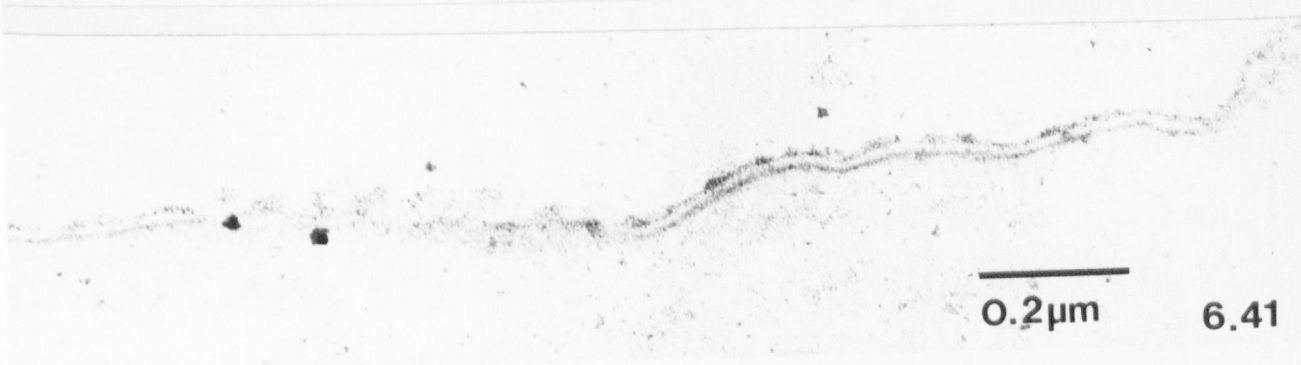

0.2µm
6.37b


0.2µm
6.37c


0.2µm
6.38


0.2µm
6.39


0.2µm
6.40


0.2µm
6.41

6.3.7 Lectins

No fluorescence was detected on the surface of third-stage larvae or adult worms. But fixed and unfixed sperm, isolated from rats, fluoresced brightly following labelling with both Con A and WGA (Fig. 6.42). Intestinal mucosa, adhering to some worms, also fluoresced, contrasting the unlabelled surface of the worm (Fig. 6.43).

6.3.8 Sodium dodecyl sulphate

The adult cuticle of *N. dubius* was apparently unaffected by the detergent SDS (Fig. 6.44). The bulk of the cuticle remained intact and the dense bars could be seen within the longtidinal ridges. But the surface of the cuticle was altered by the detergent. The surface coat was sparse and few molecules of cationized ferritin adhered to it (see Figs 6.45 and 6.6). Infective larvae survived for up to 7 days in 1% SDS.

6.3.9 β-mercaptoethanol

The reducing agent β-mercaptoethanol disrupted the cuticle of adult worms (Fig. 6.46). Only the outermost region of the cuticle and the dense bars within the longitudinal ridges remained identifiable. The surface was also affected by the reducing agent but a "tram-line" pattern measuring between 9 and 12 nm could be detected in some micrographs (Fig. 6.46). The layers of these "tram-lines" appeared to be separating.

6.3.10 Elastase

Elastase disrupted adult cuticles isolated using 1% SDS (Figs 6.47 to 6.49). Only the outer region and part of the dense bars within the longitudinal ridges remained intact after 24 hours in elastase at 37°C

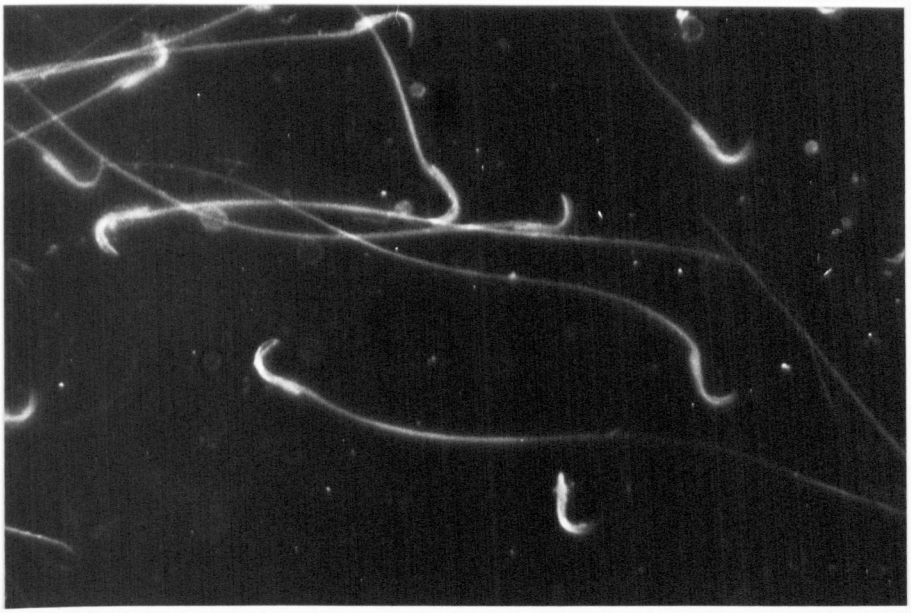


Fig. 6.42: Rat sperm fluoresces brightly following labelling with Con A conjugated to fluorescein isothiocynate (FITC). Similar results were observed when sperm were labelled with WGA-FITC. (Magnification: x730).

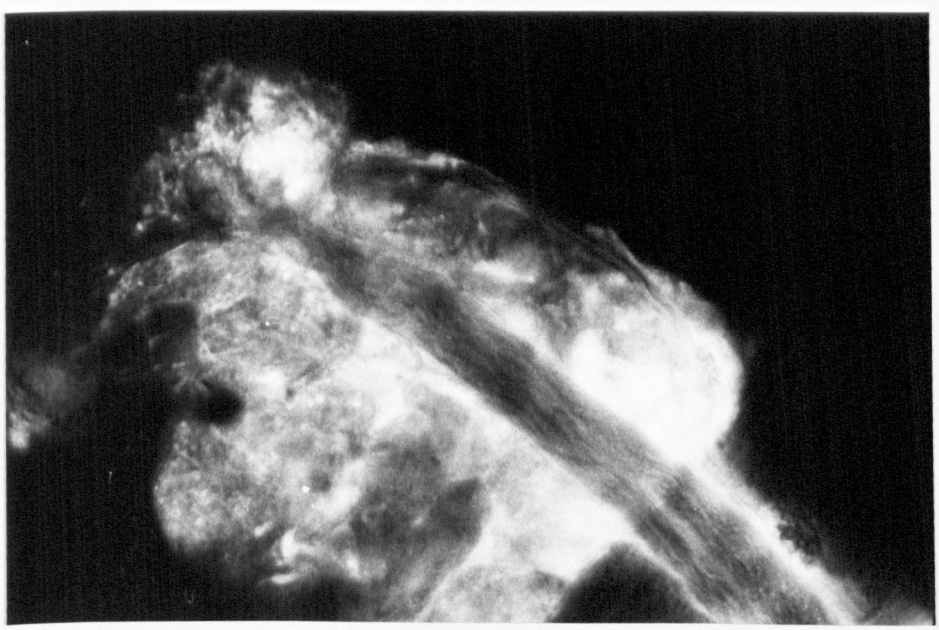
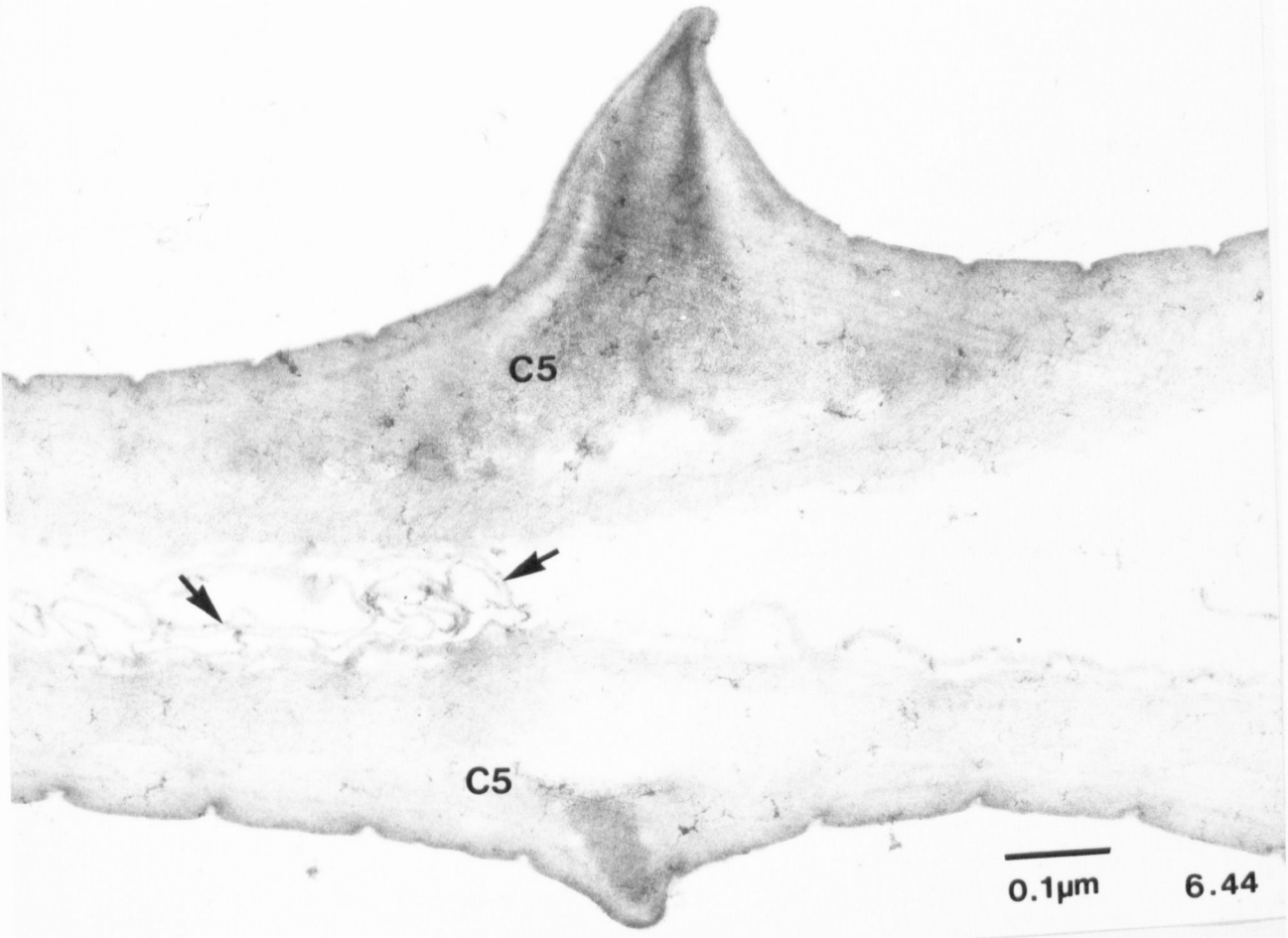


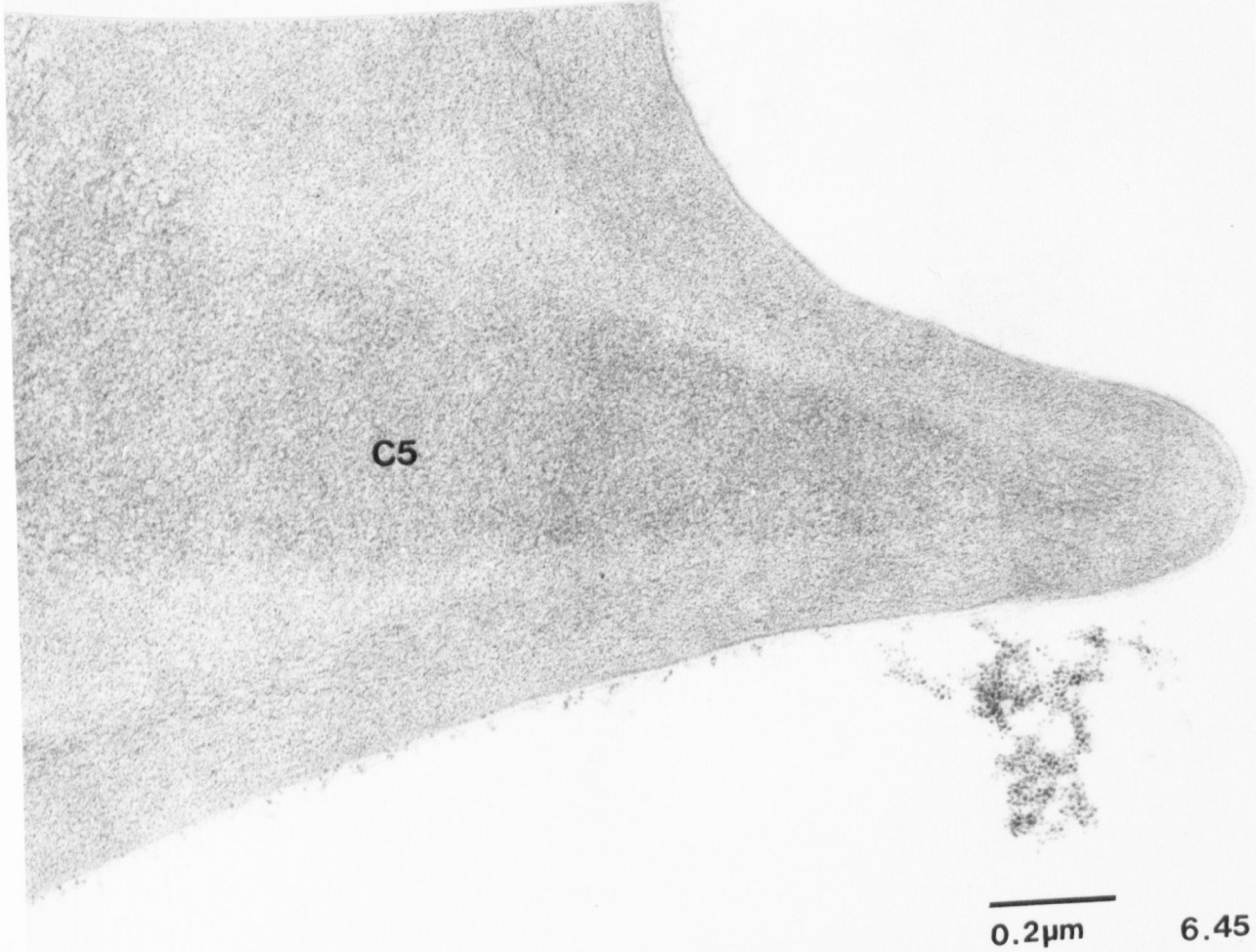
Fig.6.43: Intestinal mucosa adhering to Nematospiroides dubius fluoresced brightly following labelling with Con A conjugated to fluorescein isothiocynate (FITC), but the surface of the worm was unlabelled. (Magnification x730).

Fig. 6.44: The cuticle (C5) of an adult Nematospiroides dubius isolated using the detergent sodium dodecyl sulphate. Note that the ridges remained intact and some fragments of the internal structure can be seen (→).

Fig. 6.45: The cuticle (C5) of an adult Nematospiroides dubius isolated using sodium dodecyl sulphate and labelled with cationized ferritin. Very little cationized ferritin bound to the surface compared with untreated worms (see Fig. 6.6).

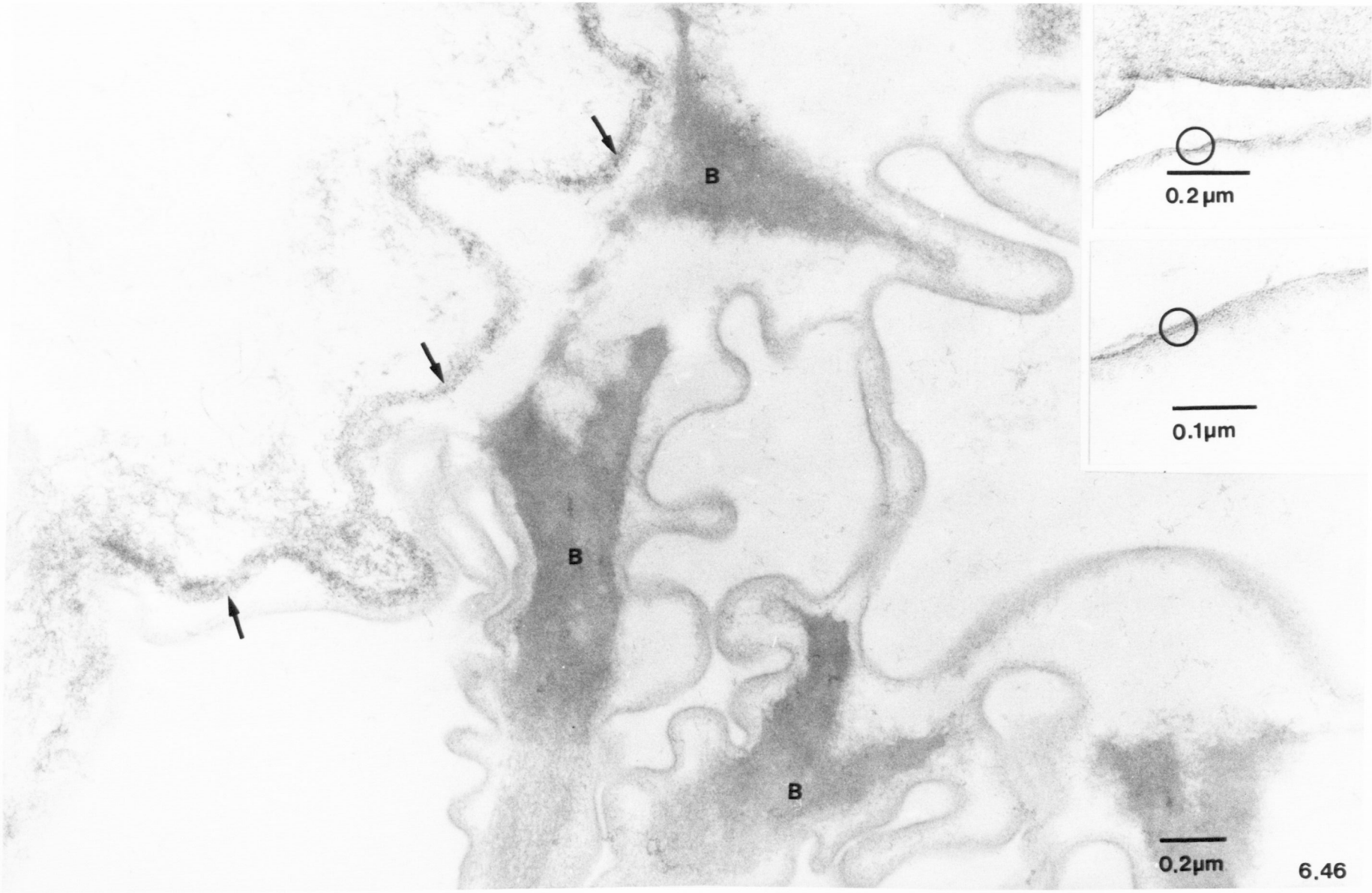


6.44



6.45

Fig. 6.46: Remains of cuticles of adult Nematospiroides dubius isolated using sodium dodenyl sulphate and exposed to the reducing agent β-mercaptoethanol. Only the outer region of the cuticle and the dense bars (B) in the longitudinal ridges remain intact. A "tram-line" pattern can be seen within the outer region (see insets). Note that fragments of some internal structures remain (→).



6.46

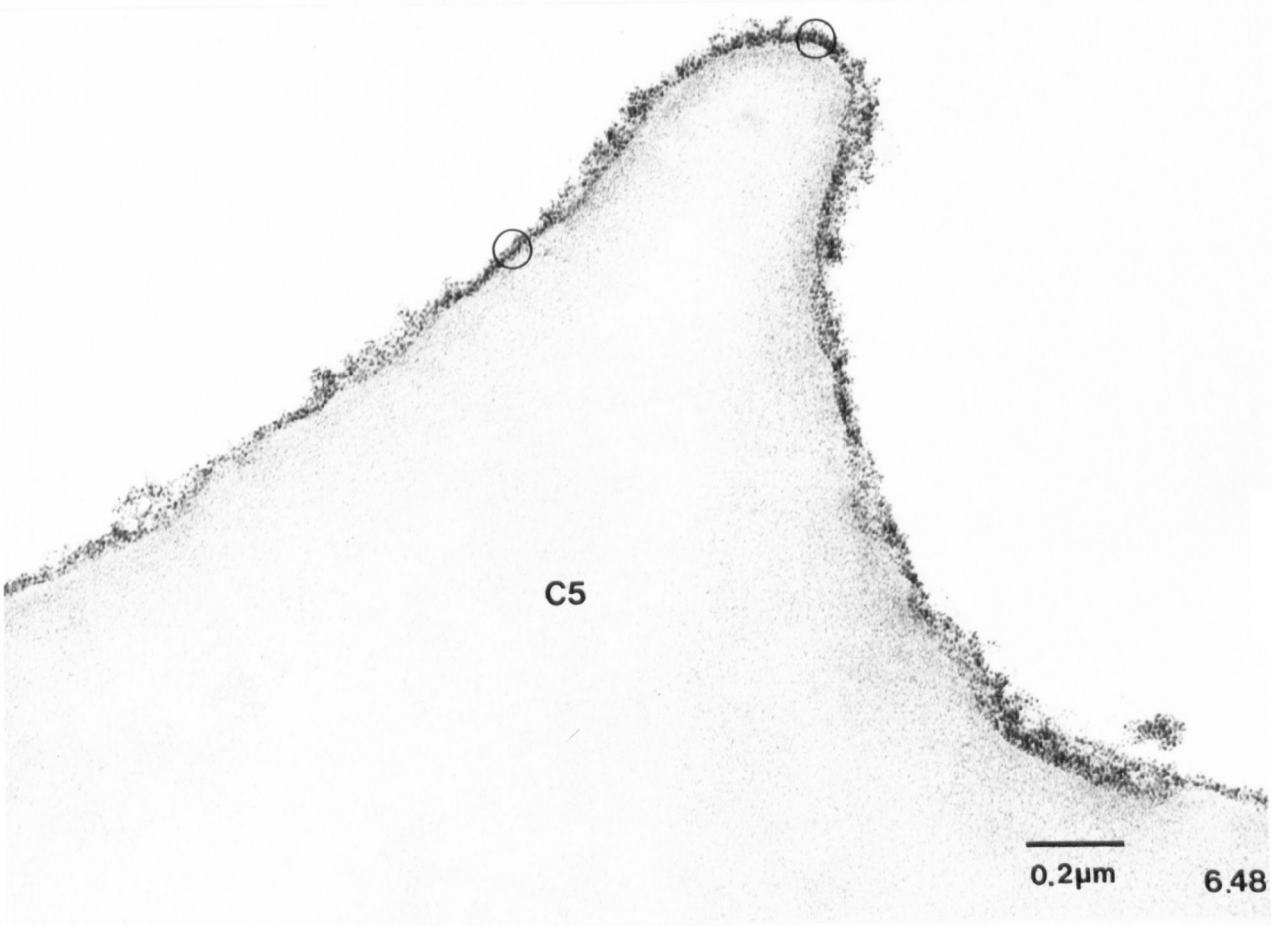
Figs 6.47 to 6.49: The remains of cuticles of adult *Nematospiroides dubius* isolated with 1% SDS and treated with elastase at 37°C.

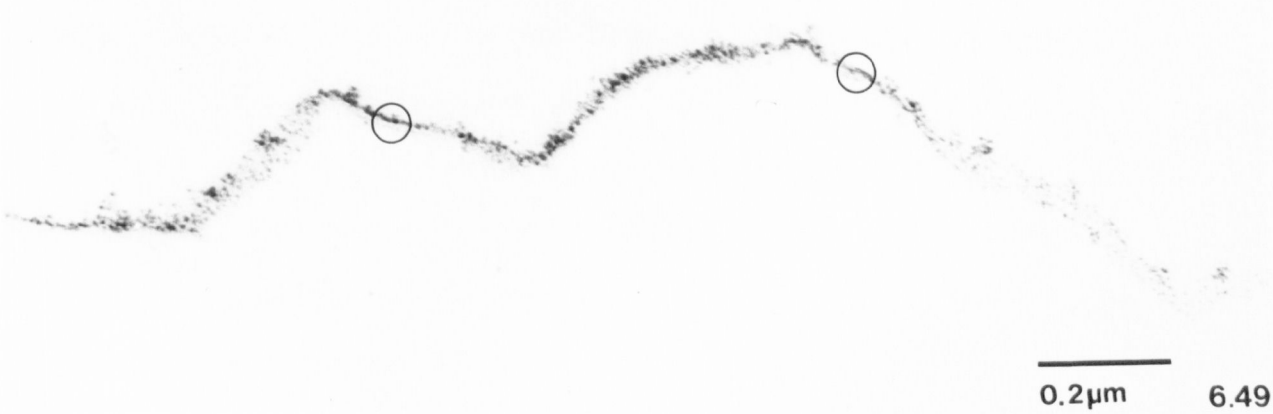
Fig. 6.47: When worms, labelled with cationized ferritin were exposed to elastase for 24 hours, only the dense bars (B) within the longutudinal ridges and the outermost region remain recognizable. Note that there is no cationized ferritin on the surface.

Fig. 6.48: The outermost region of an adult (C5) cuticle labelled with cationized ferritin, following treatment with elastase for 1 hour. In some regions a "tram-line" pattern can be detected within the cationized ferritin (circled).

Fig. 6.49: The remains of a cuticle labelled with cationized ferritin, following treatment with elastase for 24 hours. In some regions (circled) a "tram-line" pattern can be seen.


B
B
0.2µm
6.47


C5
0.2µm
6.48


0.2µm
6.49

(Fig. 6.47). The surface of the cuticle was also clearly affected by the enzyme because no cationized ferritin was present on worms that had been labelled prior to treatment with elastase (Fig. 6.47). The surface of worms re-labelled with cationized ferritin after treatment with elastase attracted a great deal of cationized ferritin and a double row of molecules was visible in some regions (Figs 6.48 and 6.49).

Elastase did not alter the susceptibility of the infective-stage stages to glutaraldehyde (Table 6.2).

6.3.11 Hyaluronidase

Hyaluronidase did not affect the permeability of the cuticle of infective-stage larvae to 3% glutaraldehyde at 22°C (Table 6.2).

6.3.12 Sodium hypochlorite

Sodium hypochlorite completely dissolved adults and third-stage larvae within a few hours.

6.3.13 Organic solvents

Adults and third-stage larvae of *N. dubius* treated with 4M guanidine-HCl died within an hour. The reagent must, therefore, have penetrated the larval cuticle, at least, but no uronic acid was detected in the supernatant. Following exposure to 4M guanidine-HCl for 7 hours, most of the adult cuticle remained intact. Only the fibre layers of the basal zone appeared to have been disrupted (Fig. 6.50). The effect of guanidine-HCl on the surface of the cuticle was highlighted by labelling treated worms with cationized ferritin (Fig. 6.50). In the main, a single, even layer of cationized ferritin adhered to the entire surface of the cuticle including the tips of the ridges. In contrast,

Pretreatment		% alive after 1 hr in 3% glutaraldehyde at 22°C
Elastase (ensheathed larvae)	elastase at 37°C (4 hrs)	13.5%
	buffer at 37°C (4 hrs)	14.7%
	Water at 37°C (4 hrs)	13.5%
Sodium periodate (ensheathed larvae)	2% sodium periodate at 30°C (1 hr)	11.8%
	water at 30°C (1 hr)	16.2%
Hyaluronidase (exsheathed larvae)	1 hr at 37°C - enzyme	18.7%
	- buffer	10%
	1 hr at RT - enzyme	33.3%
	- buffer	21%
	15 hrs at RT - enzyme	35%
	- buffer	29.1%

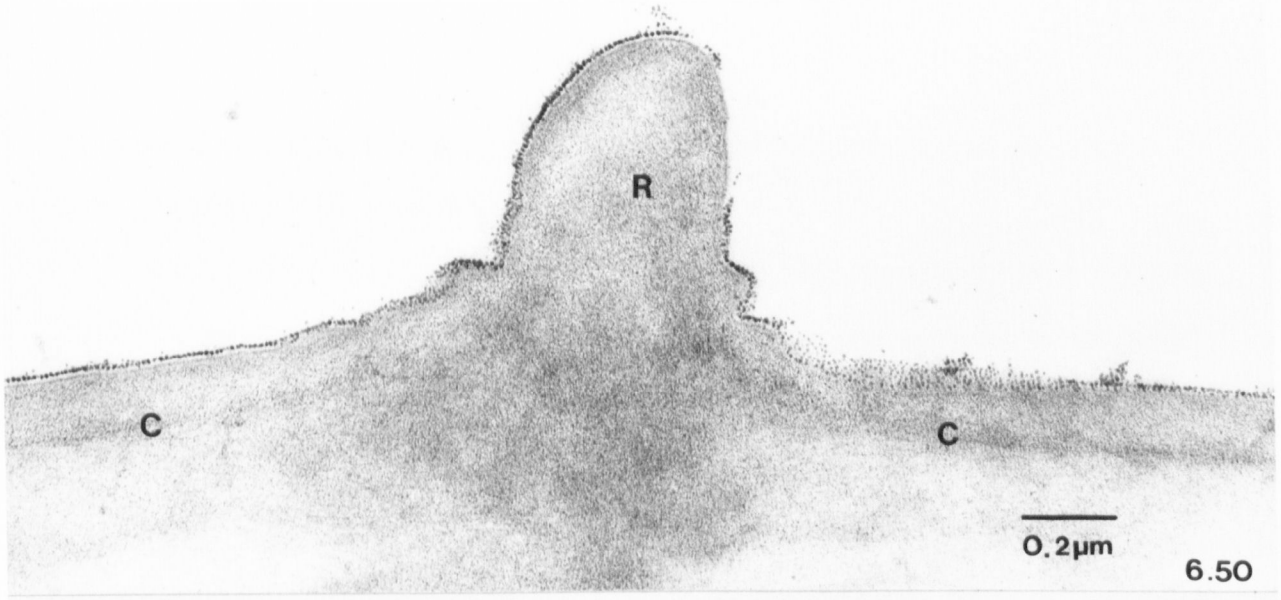
Table 6.2: The effect of elastase, hyaluronidase and sodium periodate on third-stage larvae of Nematospiroides dubius. The survival of infective larvae in 3% glutaraldehyde at 22°C was tested against untreated control worms.

Fig. 6.50: The inner layers of the cuticle of adult Nematospiroides dubius disintegrated leaving the cortical zone (C) largely intact when exposed to guanindine-HCl. Following labelling with cationized ferritin, a single row of molecules adheres to the surface of the cuticle, including the tip of the longitudinal ridge (R).

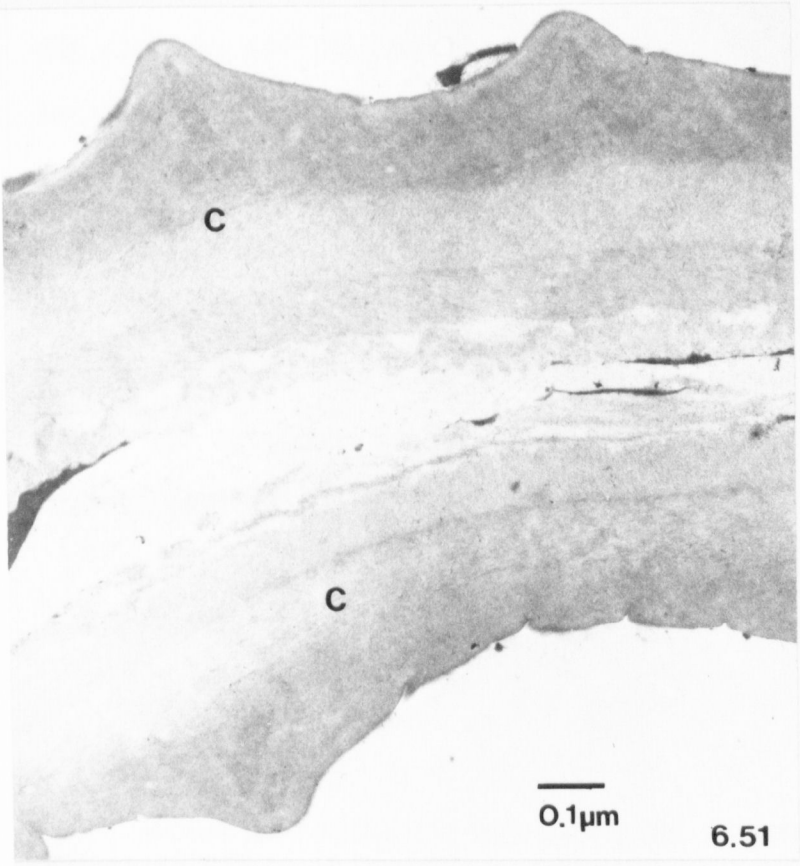
Fig. 6.51: The internal structures of adult Nematospiroides dubius dissolved when exposed to hot aqueous phenol leaving only the cuticle (C).

Fig. 6.52: A trilaminate structure (→) can be seen within a small region of the surface an adult Nematospiroides dubius exposed to hot aqueous phenol. The surface coat is absent.

Fig. 6.53: Following treatment with formic acid, the epicuticle (→) of an adult Nematospiroides dubius can be recognized but the outer line is difficult to detect.



6.50



6.51

6.52

6.53

cationized ferritin never adhered to the tips of the ridges of untreated worms (compare Figs 6.50 and 6.6).

The cuticle of adult worms emerged from hot aqueous phenol largely unscathed but the surface coat appeared to have been removed (Figs 6.51 and 6.52). The epicuticle still appeared as a trilaminate structure in a few regions, and measured between 10 and 13 nm in thickness.

A mixture of 2-chloroethanol and sulphuric acid dissolved the adult cuticle of N. dubius. Part of the worms remained after 7 hours in the mixture, but thin sections confirmed that the worm had dissolved, begining with the body wall. Within 24 hours, only a few eggs remained intact.

Following exposure to formic acid, the epicuticle, including the surface coat, measured between 12 and 25 nm in thickness and the surface coat was patchy (Fig. 6.53). A dense precipitate was visible over the top of the cuticular ridges following staining with lead citrate and uranyl acetate.

Butanol, diethyl ether and chloroform-methanol all killed infective-stages within a short time.

6.3.14 Sodium periodate

Sodium periodate did not alter the susceptibility of infective stages to glutaraldehyde at 22°C (Table 6.2).

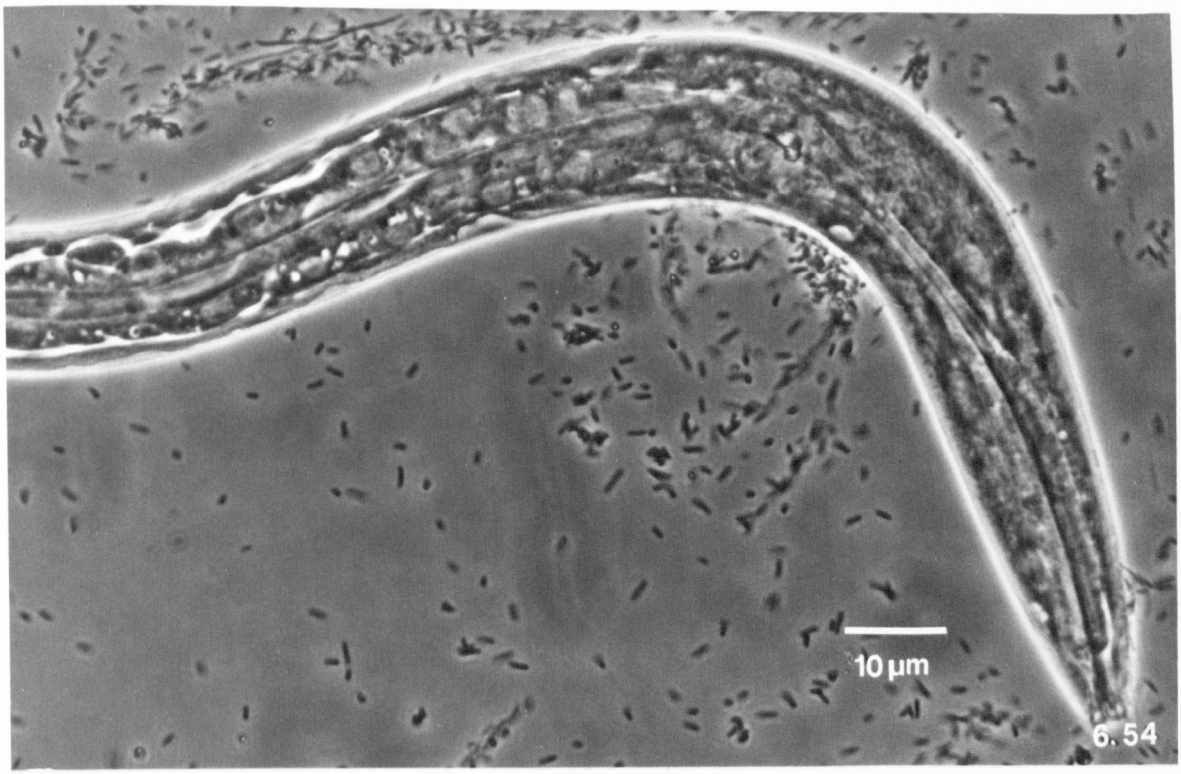
6.3.15 Association between second-stage larvae and E. COLI

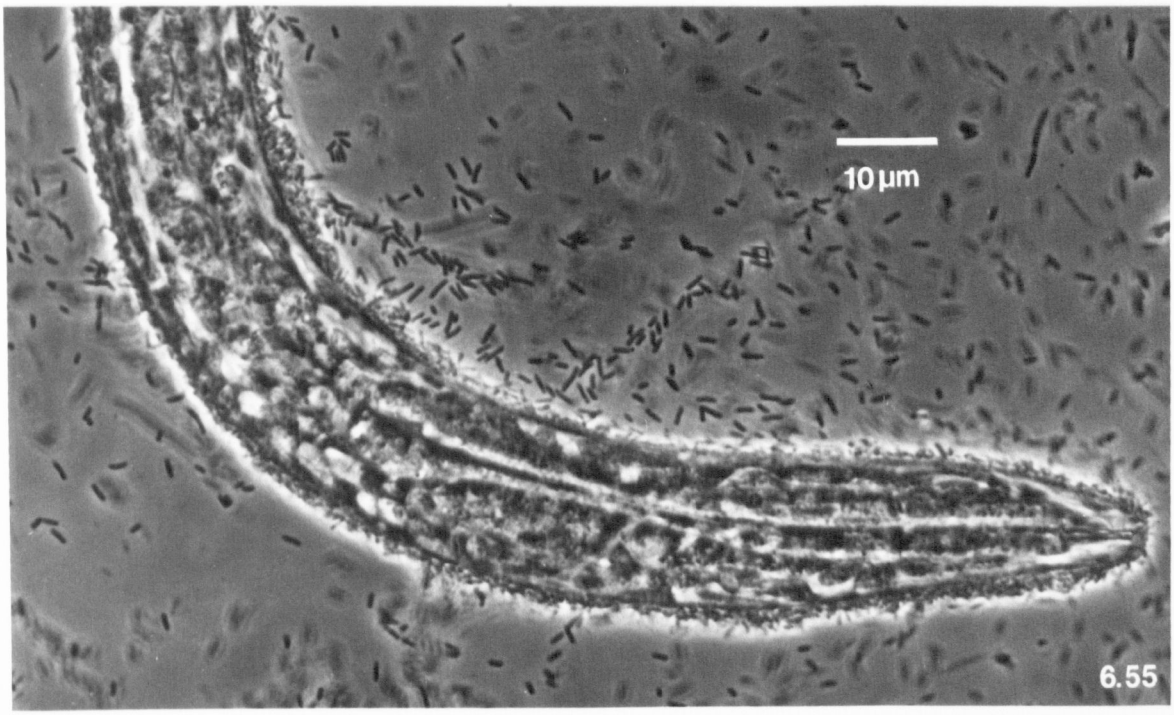
E. coli adhered to the second-stage cuticle whether the bacterial cells were dead or alive, but no E. coli were seen on first-stage worms (Fig. 6.54 to 6.56). Mammalian red blood cells did not stick to the second-stage cuticle, and pre-treatment of second-stage larvae with mannose or neuraminidase had no detectable affect on the number of

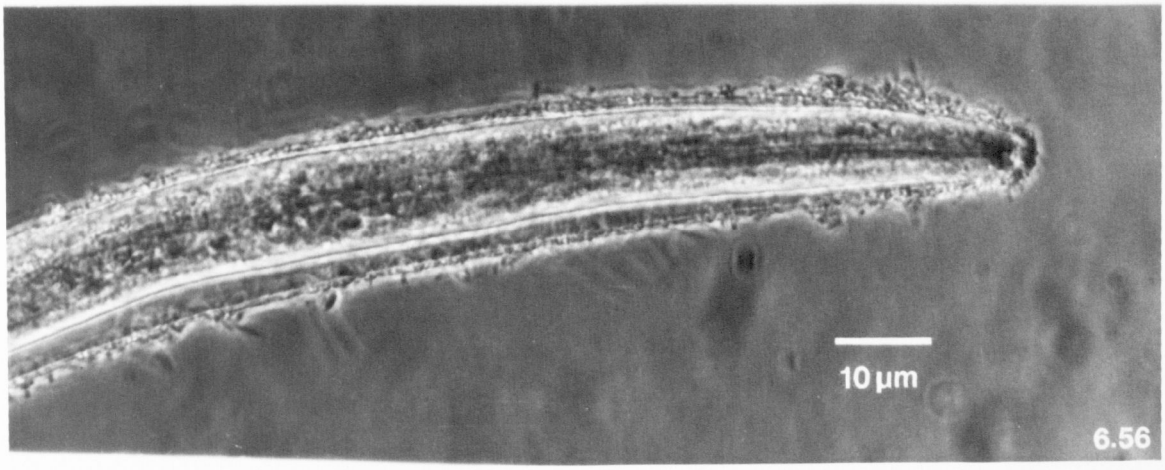
Fig. 6.54: Escherichia coli do not adhere to the cuticle of Nematospiroides dubius prior to the first moult.

Fig. 6.55: Escherichia coli adhere to the second-stage cuticle of Nematospiroides dubius.

Fig. 6.56: Some Escherichia coli remained attached to the second-stage sheath for a few days after the second (partial) moult.


10 µm
6.54


10 µm
6.55


10 µm
6.56

bacterial cells that adhered to the cuticle.

The number of E. coli adhering to the second-stage cuticle appeared to diminish as the worms developed into third-stage larvae, and few were seen adhering to infective-stage larvae when they were incubated with bacteria.

6.4 DISCUSSION

The overwhelming conclusion to emerge from this work is that the epicuticle of N. dubius varies in structure during the life cycle of this nematode. In particular, the third-stage epicuticle appears rather different from that of the other stages.

Evidence from staining indicates that the epicuticle of the adult worm probably contains some unsaturated lipid, and its susceptibility to guanidine-HCl and aqueuos phenol, suggests that protein and lipopolysaccharides might play a part in its structure. Because β-mercaptoethanol all but destroyed the epicuticle of adult worms, and SDS penetrated the third-stage cuticle, interactions between some of the major molecular components are likely to depend on non-covalent disulphide bonds.

6.4.1 Variations in the composition of the epicuticle

Variation in the composition of the epicuticle of N. dubius amongst stages probably reflects differences in the environment that these worms occupy. Similarly, Phillip and Rumjanek (1984) noted that the composition of the epicuticle of some parasitic nematodes appeared to be related to the environment in which the worms lived.

In particular, the composition of the epicuticle of third-stage

larvae was unusual compared with other stages. The free-living third-stage larvae are the only stage to be surrounded by a sheath. But after infection, the sheath is lost and these larvae are suddenly faced by a different environment in which they must survive and grow for several days, before forming a new cuticle.

The infective (C3) cuticle did not bind cationized ferritin, and as such was the only cuticle of N. dubius not to exhibit a net negative surface charge. It is of interest to note that the third-stage cuticle also altered following infectionprior to the third moult, and these changes will be discussed in Chapter 7.

The absence of net negativity on third-stage larvae is interesting because the surface of most nematodes studied to date bears a net negative surface charge (Himmelhoch et al., 1977; Murrell et al., 1982; Zuckerman et al. 1979). Only the microfilariae of D. immitis have been found not to bind cationized ferritin (Hammerberg et al., 1984), and these nematodes, like infective-stages of N. dubius, lose a sheath shortly after invading a host.

Third-stage larvae of N. dubius further differed from adult worms in that no surface coat was detected when sections were stained with uranyl acetate and lead citrate, or when worms were incubated with ruthenium red or cuprolinic blue (Figs 6.3, 6.19 and 6.33). In contrast, a filamentous coat extended from the outer leaflet of the laminate epicuticle of adult worms. The surface coat was particularly well preserved when alcian blue was included in the aldehyde fixative, appearing longer and more filamentous than when ruthenium red was used (Figs 6.14 and 6.20). Cherian et al. (1980) also found that when alcian blue-lanthanum nitrate was used to stain the surface of microfilaria of D. immitis, the reaction product was "flocculent and fibrillar" when compared with ruthenium red. Alcian blue also improves the preservation

of the surface coat of cells (Behnke, 1968; Behnke & Zelander, 1970).

While the third-stage cuticle is interesting because it undergoes an abrupt change in environment at the time of infection, the second-stage cuticle also exhibits unusual properties. The second-stage cuticle becomes detached from the cellular dermal region during the second moult, and remains surrounding the third-stage larva until a host is infected.

The second-stage epicuticle is thinner and less robust than that of any stage (see Chapter 3). And unlike the surface of adult worms and infective-stage larvae, the surface of the sheath stained intensely with cuprolinic blue suggesting that the sheath may contain sulphated proteoglycan on its surface (Figs 6.32 and 6.33).

The second-stage cuticle also differed from that of first-stage larvae, even though the two stages occupy what appears to be an identical ecological niche. Only second-stage larvae became coated with E. coli when the two were raised in a culture medium containing the bacteria. The surface of first-stage larvae remained clean.

The interaction between E. coli and the second-stage cuticle probably depends on some specific property of the bacterial and larval surface. Dead and live E. coli adhered to the worms, but mammalian red blood cells did not, indicating that not all cells are attracted to the worms.

The interaction between dead E. coli and second-stage N. dubius was unaffected by pre-treating larvae with mannose, and so is likely to differ from that between E. coli and the intestinal mucosa which is mannose-sensitive (Ofek et al., 1977). Furthermore, sialic acid does not appear to be involved in the interaction because pre-treating larvae with neuraminidase did not seem to affect the number of bacteria adhering to their surfaces. Of course, terminal sialic acid groups,

which are not involved in this interaction, may still be present on the surface of these worms.

As the second-stage cuticle separated from the epidermis during the formation of the third-stage cuticle, its surface composition appeared to alter. The number of bacteria adhering to the sheath progressively declined, and when third-stage larvae were incubated in the presence of dead E. coli, the surface of the sheath remained clean.

Presumably some changes seen in the second-stage cuticle are a consequence of its "death" after it becomes detached from the epidermis. If the sheath performs a function, it must do so as a non-living structure. For example, the sheath might prevent fungi and bacteria attaching to the surface of the third-stage cuticle. Alternatively it might prevent abrasion of the third-stage cuticle, but as the cuticle of other nematodes survives wear and tear, this does not seem to be an important consideration.

Instead it is possible that the sheath protects the larva from excess water loss. Ellenby (1968) found that ensheathed larvae of H. contortus were less susceptible to desiccation than exsheathed worms, so the sheath might act to prevent the third-stage larvae from desiccating.

The properties of the second-stage cuticle described here were noted through casual observation. No attempt was made to follow the development of the sheath. It would be of interest to compare the staining properties of the second-stage cuticle, before and after its detachment from the epidermis. Does cationized ferritin bind to the sheath, for example, and does the surface of the second-stage cuticle stain with cuprolinic blue prior to its detachment ?

Thus the composition of the epicuticle of N. dubius differs amongst stages. A difference in net negativity was observed between the

surface of third-stage larvae and that of other stages using cationized ferritin. It is tempting to correlate the unique surface properties of third-stage larvae with the fact that these free-living worms make the transition to parasitism without producing a new cuticle.

Before going on to discuss the composition of the epicuticle of N. dubius in some depth, it is appropriate to consider the significance of a net negative surface charge.

6.4.2 Negatively charged surfaces

The third-stage larvae of N. dubius are unusual in that they do not exhibit a net negative surface charge. Net negativity characterizes the surface of most nematodes studied to date (Hammerberg, et al., 1984; Himmelhoch et al., 1977; Murrell et al., 1983; Zuckerman et al., 1979).

Surface negativity appears to be a feature shared by a number of different organisms. For example, the surface of single cells, such as erythrocytes and the amoeba Naegleria gruberi exhibit a net negative charge (Danon et al., 1972; King & Preston, 1977). The surface of the envelope of bacteria also contains anionic groups (Springer & Roth, 1973), as does that of some flukes (Gress & Lumsden, 1976b; Threadgold, 1976) and cestodes (Gress & Lumsden, 1976a; Lumsden, Oaks & Alworth, 1970).

The nature of the molecular groupings responsible for the negative charge on the surface of adult N. dubius were not identified. Sialyl residues, which frequently occur on cell surfaces and have been found on nematodes (Himmelhoch & Kahane, 1983; Hudson & Kitts, 1971; Murrell et al., 1983; Spiegel et al., 1982; Spiegel et al., 1983), were located on N. dubius using neuraminidase in association with ruthenium red or cationized ferritin. Although such stains represent a relatively crude

technique, Wright (E. J., 1982) showed that neuraminidase disrupted the surface coat of spermatozoa of N. brasiliensis and N. dubius using ruthenium red as a marker. Of course, sialic acid is not the only terminal group of the carbohydrates found on cell surfaces to exhibit a negative charge. Fucose, which is a terminal group of some glycoproteins, is also negative (Kornfeld & Kornfeld, 1980).

The "function" of a negative surface has interested workers for some time. Does a net negative charge on the surface of an organism have some biological significance, or do specific molecules containing a negative terminal group have a function ? In this context it is interesting to note that some non-living surfaces also become negatively charged as they acquire a film of glycoproteins and proteoglycans when contacting an aqueous environment, whether it be within an oral cavity, or submerged beneath the sea (Baier, 1980; Characklis & Cooksey, 1983, Marshall, 1980).

One suggestion is that intense negativity provides a protective repelling force against approaching cells. The parasite Leishmania donovani, for example, has a highly charged surface which might repel host cells (Dwyer, 1977). Another way in which "negative charge" may protect a surface is by blocking enzymes. The negatively charged carbohydrate moiety of some glycoproteins may protect the protein part of the molecule from non-specific cleavage by enzymes (Kornfeld & Kornfeld, 1980). For example, the carbohydrate component of the glycoprotein, fibronectin, is thought to protect the molecule against proteolytic attack (Yamada et al., 1980), and there is evidence that terminal sialic acid groupings prevent certain glycoproteins from being absorbed by liver cells (Morell, Gregoriadis, Scheinberg, Hickman & Ashwell, 1971; Van den Hamer, Morell, Scheinberg, Hickman & Ashwell, 1970).

An attempt was made to study the distribution of negative charge on the surface of adult N. dubius labelled with cationized ferritin using microprobe analysis (see Appendix 1). Although the counts of the amount of ferritin fluctuated too widely to draw any conclusions for the most part, little or no ferritin was consistently detected on the bursa of males (see Appendix 1). Similarly, diminished binding of cationized ferritin was found in the tail region of C. briggsae (Himmelhoch et al., 1977). The significance of this observation in unclear. It is possible that iron associated with haemoglobin within the cuticle was also detected by the microprobe, and this contributed to the inconsistent results.

In retrospect, however, it was decided that the microprobe analysis probably is not suited for establishing meaningful data about the surface of nematodes. For the microprobe to detect ferritin in this study, counts had to be made over an area of the nematode surface. But it seems likely that if differences in the composition of the epicuticle are to have biological significance, they would probably occur at the molecular level, and so would be overlooked by this method. The receptors believed to be involved in chemotaxis in some nematodes, for example, are thought to be extremely localized (Zuckerman, 1983).

6.4.2 Composition of the epicuticle

A major portion of the epicuticle of adult N. dubius, at least, seems to be composed of a bilayer of lipid (see also Chapter 5). Proteins, some conjugated to carbohydrate in the form of glycoproteins, are also probably important. But proteoglycans do not appear to be of fundamental importance to the structure of the epicuticle of N. dubius.

The interactions between the molecular components of the epicuticle of N. dubius seem to be non-covalent and hydrophobic because

some organic solvents, and the detergent SDS, penetrated the third-stage cuticle and altered the adult epicuticle (Figs 6.42 and 6.43). Organic solvents and detergents are thought to attack the hydrophobic interactions within plasma membranes (Gel'man et al., 1975).

Although SDS penetrated the third-stage cuticle, its effect was not immediate. Some larvae survived in the detergent for up to 7 days. Moreover, SDS did not destroy the trilaminate structure of the adult epicuticle, but did alter its surface composition (Fig. 6.45). Similarly, the outer membrane of bacteria can also survive exposure to SDS (DiRienzo, Nakamura & Inouye, 1978). Further similarities between the epicuticle of adult N. dubius and the envelope of bacteria were discussed in Chapter 5.

Interactions between molecules making up the epicuticle of N. dubius may also depend on disulphide bonds. The epicuticle was largely disrupted by a strong reducing agent, although some remaining fragments of the epicuticle appeared trilaminate (Fig. 6.46).

Similarly Clark et al. (1982) found that molecules making up the epicuticle of T. spiralis were susceptible to reduction, and were therefore probably dependent on disulphide bonds. Using electrophoresis, they concluded that the important disulphide bonds occurred within the protein molecules, and not between them. In contrast, the epicuticle of C. elegans and S. ratti survived exposure to a detergent and reducing agent suggesting that neither disulphide bonds, nor hydrophobic interactions were important in maintaining the integrity of the epicuticle in this species (Cox et al., 1981a; Murrell et al., 1983).

The only part of the internal structure of N. dubius to survive treatment with β-mercaptoethanol was what appeared to be part of the pseudocoelomic membranes which surround the muscle. This observation is

consistent with conclusions of Dawson (1960) who found that these structures were not susceptible to collagenase.

The importance of lipid in the composition of the epicuticle is evidenced by its susceptibility to lipid solvents. Lipid solvents penetrated the infective cuticle in a short time, verifying earlier findings (Arthur & Sanborn, 1969; Rogers, 1961).

Further evidence for the presence of lipid in the epicuticle comes from staining with tannic acid. Tannic acid improved the contrast of the adult epicuticle and enhanced the electron-density of the outermost layer of the third-stage cuticle. (The dense precipitate that was sometimes apparent outside the third-stage cuticle is probably an artefact from a reaction between osmium tetroxide and tannic acid [Hayat, 1981]).

The mechanism by which tannic acid reacts with membranes is not fully understood, but it is thought to stabilize the ordered structure of phosphatidyl choline systems by binding with osmium tetroxide and reactive sites on the choline, as well as by cross-linking proteins (Gustavsen, 1949; Kalina & Pease, 1977). Tannic acid increases the contrast of intra- and extracellular structures, including cell membranes, by acting as a mordant between osmium tetroxide and lead staining (Futaesaku, Mizuhira & Nakamura, 1971; Kalina & Pease, 1977; Rodewald & Karnovsky, 1974; Simonescu & Simonescu, 1976a, 1976b; Wagner, 1976). Thus staining with tannic acid suggests that lipid is present in the epicuticle.

Evidence that double bonds are present within the lipid of the adult epicuticle comes from staining with ruthenium red (see also Chapter 5). Blanquet (1976a; 1976b) noted that staining with ruthenium red was dependent on the number of double bonds in the outer half of a lipid matrix, as well as on the acidic groups. He proposed that during

the staining of plasma membranes, colloidal osmium dioxide forms when osmium tetroxide reacts with the unsaturated lipid. The ruthenium red molecule then recharges the colloidal osmium dioxide to produce a positive colloid at the cell surface.

Tannic acid and ruthenium red stained the inner and the outer lines of the epicuticle with equal density. In comparison, alcian blue or uranyl acetate and lead citrate stained the inner line with more density than the outer one (Figs 6.20, 6.14 to 6.17, Figs 6.23 and 6.24). The significance of this difference is unknown.

Proteins also seem to be involved in the molecular structure of the epicuticle of N. dubius, sometimes conjugated to carbohydrates in the form of glycoprotein. Guanidine-HCl, a strong solvent of proteins (Zahler & Niggli, 1977), penetrated the cuticle of non-feeding infective larvae and killed them within an hour. It also affected the surface of the adult cuticle, but the trilaminate structure of the epicuticle appeared to remain intact (Fig. 6.50). Similarly, guanidine-HCl did not remove the surface coat or negative charge from the surface of S. ratti (Murrell et al., 1983). Formic acid, which disrupts H-bonds and so has the capacity to disocciate the lipid and protein components of membranes (Zahler & Niggli, 1977), also affected the surface of N. dubius (Fig. 6.53).

The glycoprotein, elastin, which is common within the extracellular matrix of multicellular organisms, appears to play an important role in the structure of the cuticle of N. dubius. When the cuticles of adults were exposed to elastase, most of the cuticle was degraded and the surface was modified, as demonstrated by its reaction with cationized ferritin. But the trilaminate appearance of the epicuticle seemed to survive. Elastase did not affect the permeability of the third-stage cuticle when applied to the outside of these

animals. In contrast, Cox et al. (1981b), found that elastase hydrolyzed the outer portion of C. elegans, and Murrell and Graham (1982) found that elastase affected labelled antigens on the surface infective-stage larvae of S. ratti.

Elastin was implicated in forming part of the inner layers of the adult cuticle of N. dubius. Not only was the basal zone degraded by elastase, but it also stained with tannic acid (Fig. 6.24). Tannic acid is thought to form cross-links with protein and elastin (Gustavson, 1949; Locke & Huie, 1975).

Apart from being conjugated to proteins, carbohydrate might also occur in association with lipid in the form of lipopolysaccharides. Aqueous phenol, a solvent for lipopolysaccharides and glycoproteins, completely dissolved the surface coat from adult worms and the trilaminate epicuticle could only be detected in small areas, often in the folds of the cuticle produced as the worm coiled (Figs 6.51 and 6.52).

There was no evidence that α-D-mannose/α-D-glucose, or N-acetyl-D-glucosamine/N-acetyl-D-neuraminic acid/N-acetyl-D-galactosamine, occurred on the surface of infective-stage larvae or adult N. dubius using FITC-conjugated lectins. However, the absence of fluorescence coes not mean that these carbohydrates are not present. It is possible that the sugars occur in small numbers. Zuckerman and Kahane (1983), for example, suggested that radiolabelled lectins are a more sensitive tool for analyzing surface carbohydrates where the sugars are few in number and scattered. They found that 3 to 9% of the surface of C. elegans labelled with radiolabelled WGA, but no fluorescence was detected if WGA-FITC was used instead.

It seems unlikely that the lack of fluorescence was caused by labelled surface components being shed from the surface before being

viewed. Specimens were always removed from the lectin, washed, mounted and viewed within 5 minutes. Shedding of antigens from the surface of other species of nematode takes some hours (Maizels et al., 1983; Smith et al., 1981).

Some indication that carbohydrates may be present on the surface of adult N. dubius comes from staining with bismuth subnitrate. A fine electron-dense precipitate appeared to be associated with the epicuticle when sections were oxidized with periodic acid and then stained with bismuth subnitrate, suggesting that aldehyde groups are present (Figs 6.37 and 6.38). Where periodic acid was omitted, the outer portion of the epicuticle was never more dense than the inner one (Figs 6.39 to 6.41). Although staining with bismuth subnitrate indicates that carbohydrate might be present on adult N. dubius, I would need confirmation of this result using a different method because the electron micrographs are not considered conclusive.

Proteoglycan does not appear to be a major component of the epicuticle of N. dubius. Proteoglycan molecules, such as hyaluronate, which form an important part of the extracellular matrix, can be secreted on to the surface of the plasma membrane following synthesis on the inner face of the membrane (Prehm, 1984), but such molecules do not appear to play an important role in the epicuticle of N. dubius.

The third-stage and the adult cuticle did not stain intensely with cuprolinic blue. In contrast, the surface of the sheath was electron-dense following exposure to the dye. Because the sheath is a non-living structure, perhaps it is not surprising that it differs in composition from the third-stage and adult cuticles. Similarly, Murrell et al. (1983) also suggested that a sulphated proteoglycan, similar to keratan sulphate, were present on the surface of S. ratti.

Furthermore, periodate, which is thought to have the capacity to

degrade proteoglycan by splitting glycol groups, had no effect on the permeability of the third-stage cuticle to glutaraldehyde (Scott, Tigweel, Phelps & Nieduszynski, 1976; Scott, Tigwell & Saydera, 1972). Thus, proteoglycans may be present on the surface of second-stage larvae, but they probably do not form a fundamental part of the composition of the epicuticle.

Although the epicuticle of N. dubius did not stain intensely with cuprolinic blue, the central portion of the longitudinal ridges of the adult cuticle did accummulate the dye. A dense precipitate was associated with the inner region of these ridges suggesting that proteoglycan is present.

Clearly the molecular composition of the epicuticle of nematodes is little understood. Fundamental differences in structure have been recorded for different species. For example, the cuticle of adult N. dubius was not permeable to cationized ferritin, and no pores were detected within the cuticle. On the other hand, Poinar & Hess (1977) found that ferritin was taken up by the cuticle of R. culicivorax. Hexagonal subunits similar to those found by Poinar & Hess (1977) were observed in the cuticle of N. dubius (see Figs 6.24 and 4.16) but these were interpreted as being the structural "building blocks" of the cuticle and not pores.

Similarly, disulphide bonds are thought to be important in maintaining the structure of the epicuticle of T. spiralis and N. dubius, but not C. elegans (Clark et al., 1982; Cox et al., 1981b). And sodium hypochlorite completely dissolved N. dubius but had no apparent effect onthe surface of S. ratti (Murrell et al., 1983). It is not known whether these differences represent true differences between species, or whether the techniques that have been used are unreliable.

This study of the composition of the epicuticle of N. dubius has

proved valuable. We now know something about the adult cuticle of N. dubius, and that it is rather different from that of third-stage larvae. Thus, the epicuticle of adult N. dubius appears to depend on a bilayer of some sort of lipid which is associated with protein, sometimes present in the form of glycoproteins. Interactions between the major components are probably non-covalent.

On the other hand, the composition of the epicuticle of the infective-stage appears quite different. Although susceptible to guanidine-HCl and lipid solvents, the epicuticle of the worm did not split when freeze-fractured (see Chapter 5). Furthermore, the surface of the infective cuticle did not stain intensely with the stains used on the surface of the adult cuticle. Nor did it exhibit a net negative charge.

Nevertheless, this study must be regarded as being a preliminary investigation. The idea of immersing a whole animal in a chemical and then observing its effect with an electron microscope is relatively crude, although it has the advantage of being able to visualize the action of the chemical. Few other techniques are avalable, and these have limitations (Parkhouse et at., 1981; Bashong & Rudin, 1982; Marshall & Howells, 1985), which makes interpretation uncertain.

CHAPTER 7

CHANGES IN FREE-LIVING LARVAE OF N. dubius ASSOCIATED WITH THE TRANSITION TO PARASITISM

7.1 INTRODUCTION

Parasitic life histories are discontinuous in time and space (Davey, 1982). A parasitic species must be capable of moving from one host to another, as well as surviving in markedly different environments.

One way that parasitic nematodes "bridge" the gap between two different environments is by forming a resistant infective-stage (Rogers & Sommerville, 1963; 1968). In these stages, the gene-set which directs the development of the first parasitic stage is suppressed. When an appropriate signal is received from the host, the inhibition of the gene-set is lifted and development resumes (Rogers & Petronijevic, 1982). Free-living nematodes may also form a resistant dispersive stage, the "dauer larva", which seems similar to the infective-stage.

When moving between different environments, both free-living and parasitic nematodes undergo changes. The cuticle may alter, as may the way in which the nematode behaves (Davey, 1982; Rogers & Petronijevic, 1982). Of course, metabolic changes also occur but these will not be discussed here (Bryant, 1982; Rogers & Petronijevic, 1982).

Soon after some parasites invade a new environment, the cuticle undergoes ultrastructural changes. Wright (1982) noted that the surface of infective first-stage larvae of T. spiralis altered within 6 hours of the worms entering the columnar cells of the host intestine, and Bird (1968) found fundamental changes in the cuticle of infective-stage

larvae of M. javanica within a few days of the worms invading a new host.

Formation of a resistant dauer larva can also involve ultrastructural changes to the cuticle (Cassada & Russell, 1975; Kondo & Ishibashi, 1978). The epicuticle of some species of dauer larvae, for example, is thicker than that of propagative forms. And because the dauer larvae can survive prolonged exposure to certain chemicals, their epicuticle is apparently less permeable (Bird & Buttrose, 1974; Cassada & Russell, 1975; Kondo & Ishibashi, 1978).

These ultrastructural changes of the cuticle, which presumably play a role in the survival of nematodes, need not be accompanied by the formation of a new cuticle. Indeed, because nematodes can change in this way, it seems that whatever the reason for moulting, it is not required to alter the structure of the cuticle.

Another way a parasite may respond to a changing environment, is by changing its behaviour. Bird and Stynes (1981) could distinguish freshly hatched worms of A. agrostis from dauer larvae because the former were more tightly coiled and moved more slowly than the dauer larvae. Similarly, the behaviour of first-stage larvae of T. spiralis alters when the worms enter a new environment (Despommier, 1982). Larvae taken from host muscle cells exhibit coiling-uncoiling movements but once the worms, still in the same developmental stage, enter the small intestine of the host, their movement is wave-like.

Casual observations suggest that the free-living third-stage larvae of N. dubius move in a different way to those that have been in a host for some time. The latter move by coiling and uncoiling, but the infective-stages use undulatory waves to move.

Why these changes in the behaviour of nematodes occur is not known, but it seems possible that movement might be affected by alterations to the body wall. Several authors have implicated the

cuticle in the movement of nemtodes. Wisse and Daems (1968) proposed that the undulatory movements made by small nematodes were produced by the muscles acting against the cuticle. Alternatively the cuticle, in conjunction with the internal pressure of the worm, might provide the force against which the muscles act (Harris & Crofton, 1957; Seymour, 1973; Wright, 1968). So changes in the structure of the cuticle, and its attachment, could alter the way in which nematodes move.

The cuticle of nematodes, therefore, not only serves as a boundary between animal and environment, but is also implicated in their movement. In light of these observations, the transition of N. dubius from a free-living worm to one that is parasitic, was examined. Of particular interest were any changes in the ultrastructure of the third-stage cuticle as it is confronted by a new environment. The way in which larvae moved, before and after infection, was also studied to see if structural changes and patterns of movement could be correlated.

7.2 MATERIALS AND METHODS

Mice were infected with 500 third-stage N. dubius larvae as described in Section 2.2.1. Late fourth-stage larvae were collected as described in Section 2.2.2.

To study the third-stage larva prior to the third moult, worms were removed from the host at 24, 48, 72, 83, and 96 hours after infection (see Section 2.2.2). Larvae at different stages of development were photographed under a Wild M20 light microscope. Others, some labelled with cationized ferritin (see Section 6.2.1), were fixed and embedded for transmission electron microscopy (see Section 2.3). In addition, some worms removed from the host 24 and 72 hours

after infection were labelled with FITC-conjugated Con A and WGA (see Section 6.2.1).

An indication of the growth rate of N. dubius after infection was obtained by measuring the lengths of : 50 infective-stage larvae, 50 larvae removed from mice 64 hours after infection, and 10 larvae removed from a host 96 hours after infection. Larvae were partially straightened by fixing them in a paraformaldehyde-glutaraldehyde fixative (Glauert, 1974) at 60°C for 15 minutes.

Movement of larvae was studied by modifying existing methods (Rode & Staar, 1961; Sandstedt, Sullivan & Schuster, 1961; Wallace, 1969). Worms were removed from mice 6, 18, 24, 48, 72 and 96 hours after infection. Ten worms from each infection and 10 infective-stage larvae were washed in 0.85% saline and placed individually on 4% agar plates (Difco Bacto-Agar, Detroit, USA) in a drop of saline. Plates were no more than 4 mm deep and had been allowed to stand overnight at room temperature. Once the excess moisture surrounding the larvae was removed, using either a capillary tube or millipore filter paper, active larvae began to move across the agar leaving a trail in the surface. Plates were left at 26 to 30°C for 150 minutes, after which time they were checked for signs of activity. Worms were then killed by inverting the agar plate over a few drops of chloroform for 2 to 3 minutes (Ward, 1973).

Tracks left by larvae in agar were classified according to whether they were composed of : a sinusoidal wave-like pattern, a series of unpredictable wriggles, or a combination of the two. Some worms were active but did not move over any distance, leaving a pit imprinted in the agar. An indication of the distance each worm travelled in the alloted time was obtained by placing the plates over a mm^2 grid. The number of squares containing tracks was counted but as it was impossible

to distinguish overlapping tracks, no attempt was made to count the number of times a worm crossed a given mm^2. The tracks were photographed under a Wild M5 binocular microscope fitted with a trans-illuminated base in which the light was slightly off axis. A Wild MPSS1 camera was used.

This study of the movement of third-stage larvae of N. dubius was carried out twice.

The wave patterns left by larvae at different stages of development were compared. Fifty measurements of wavelength and amplitude were recorded from photographs of tracks left by : 4 infective-larvae, 4 larvae exposed to a host for 6 hours, 2 larvae exposed to a host for 18 hours, and 2 larvae exposed to a host for 48 hours. Because just 1 out of the 10 larvae that had been exposed to a host for 72 hours left a wave-like pattern in agar, only 20 measurements could be made from this treatment.

In an attempt to relate ultrastructural alterations in the body wall of third-stage larvae to changes in their movement, larvae taken from mice 54 hours after infection were placed on agar plates, as described above, and left overnight at 37°C. Worms that were still alive 18 hours later were fixed, uncut, and prepared for transmission electron microscopy (see Section 2.3). Once embedded, the lengths of these worms were measured. Thin sections were examined from 18 larvae which had moved leaving a variety of tracks.

7.3 RESULTS

7.3.1 Behaviour before and after infection

The third-stage larvae of N. dubius grew as they developed in the

host. The mean length of infective larvae, not including the enveloping second-stage cuticle, was 0.49 ± 0.002 mm ([± standard error] range: 0.42 to 0.50 mm). Within 64 hours of infection, the mean length was 0.63 ± 0.02 mm (range: 0.3 to 1.02 mm), which was significantly different from that of infective-stages ($P < .02$, see Appendix 2). In comparison, the mean length of fourth-stage larvae removed from the host 96 hours after infection was 1.5 ± 0.09 mm (range: 1.27 to 2.1 mm).

Differences between infective larvae and those removed from a host 24 to 96 hours after infection are illustrated in Fig. 7.1.

Apart from these morphological differences, changes also occurred in the behaviour of larvae after infection (Table 7.1). Of the larvae placed on agar plates, only the infective-stages were still moving after 150 minutes at 26 to 30°C. In contrast, every post-infective worm had stopped moving within this time. While infective-stage worms covered an average distance of nearly 50 mm, larvae that had been in a host for 6 hours moved through significantly less distance ($.01 > P > .002$, see Appendix 3). The capacity of the worms to travel any distance across the agar appeared to be related to the length of time they had been exposed to the host (Table 7.1). If removed from the host 6 hours after infection, for example, worms moved about 22 mm, and late third-stage worms covered little or no distance.

Once exposed to a host for 18 hours or longer, larvae assumed a coiled position when inactive (Figs 7.1 and 7.2). Infective-stage worms and those recovered from the host after 6 hours, on the other hand, invariably stopped moving in a straightened position.

Infective stages, and larvae exposed to a host for 6 hours, left a sinusoidal wave pattern in the surface of the agar (Fig. 7.2a, Table 7.1). These larvae frequently reversed and changed direction but

Fig. 7.1: Light micrographs of third-stage larvae of Nematospiroides dubius: (a) prior to infection, (b) 24 hours after infection, (c) 48 hours after infection and (d) 72 hours after infection. Extensive changes are apparent in worms exposed to a host for about 48 hours. Two excretory cells, each with a single large nucleus (→), can be seen. The genital primordium (G) can be distinguished in (c) and (d). For comparison, a fourth-stage larva (e) removed from the host 96 hours after infection is shown. Note that only the infective-stage larva is straight.

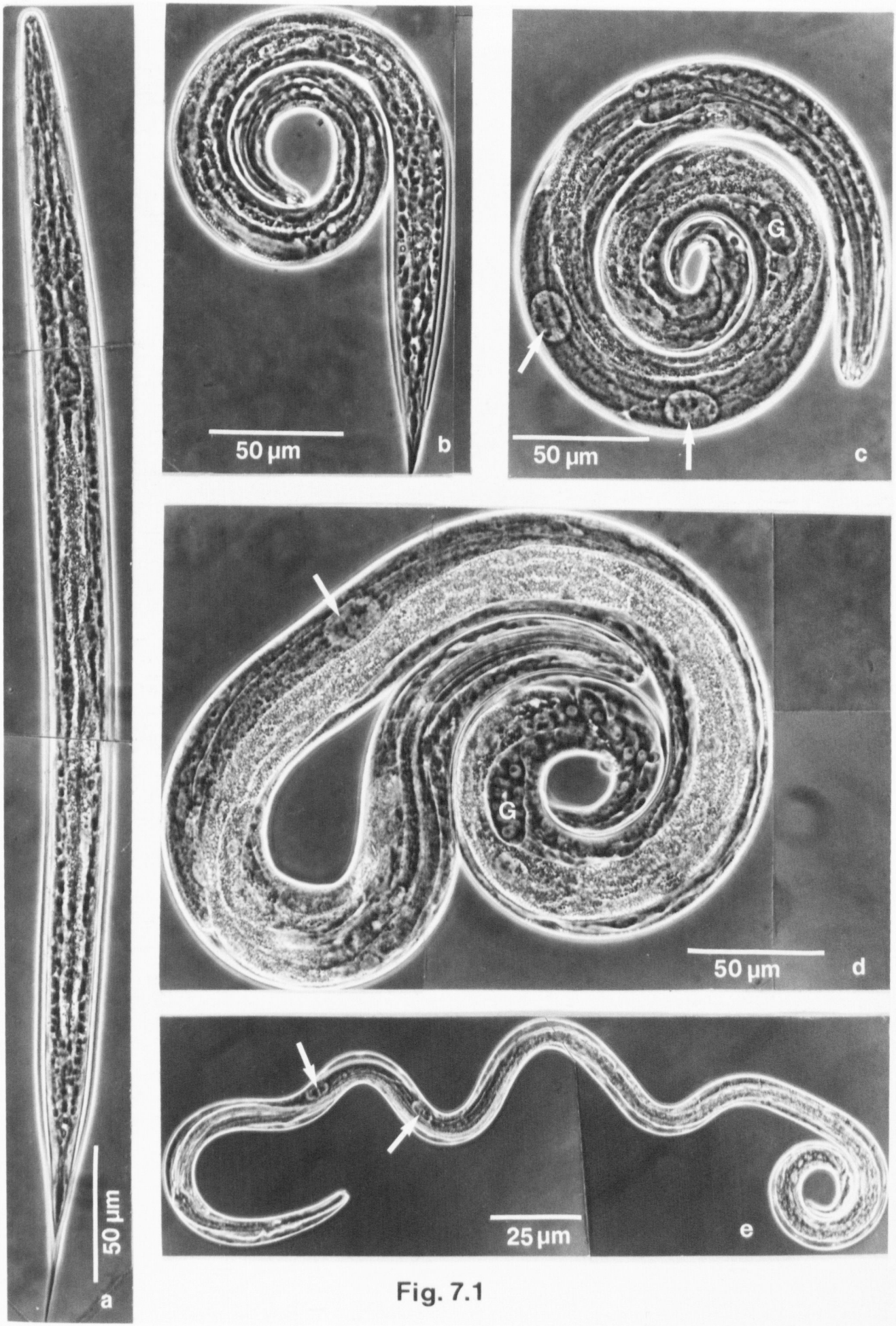


Fig. 7.1

Fig. 7.2 (a to f): Light micrographs of tracks left in agar plates by third-stage larvae of Nematospiroides dubius at different stages of development. Note the coiled worm (W) in (b), (d), (e) and (f).

Fig. 7.2(a): An example of the sinusoidal wave pattern left by infective larvae. Tracks made by larvae exposed to the host for 6 hours are similar. Some places where the worm has changed direction are indicated by (→).

Fig. 7.2(b): Tracks left by worms after 18 to 24 hours in the host. Although the wave-like pattern predominates, less distance was covered.

Fig. 7.2(c): After 24 to 48 hours in the host, a wave pattern is hardly recognizable. The number of wriggles (→) has increased.

Fig. 7.2(d): After about 48 hours in the host, movement of larvae is characterized by a series of wriggles.

Fig. 7.2(e): Some larvae do not move across the agar after they have been in the host more than 48 hours. Although active, only a few wriggles are recorded in the agar.

Fig. 7.2(f): Late third-stage larvae moved very little. A pit can be seen in the surface of the agar around the worm.

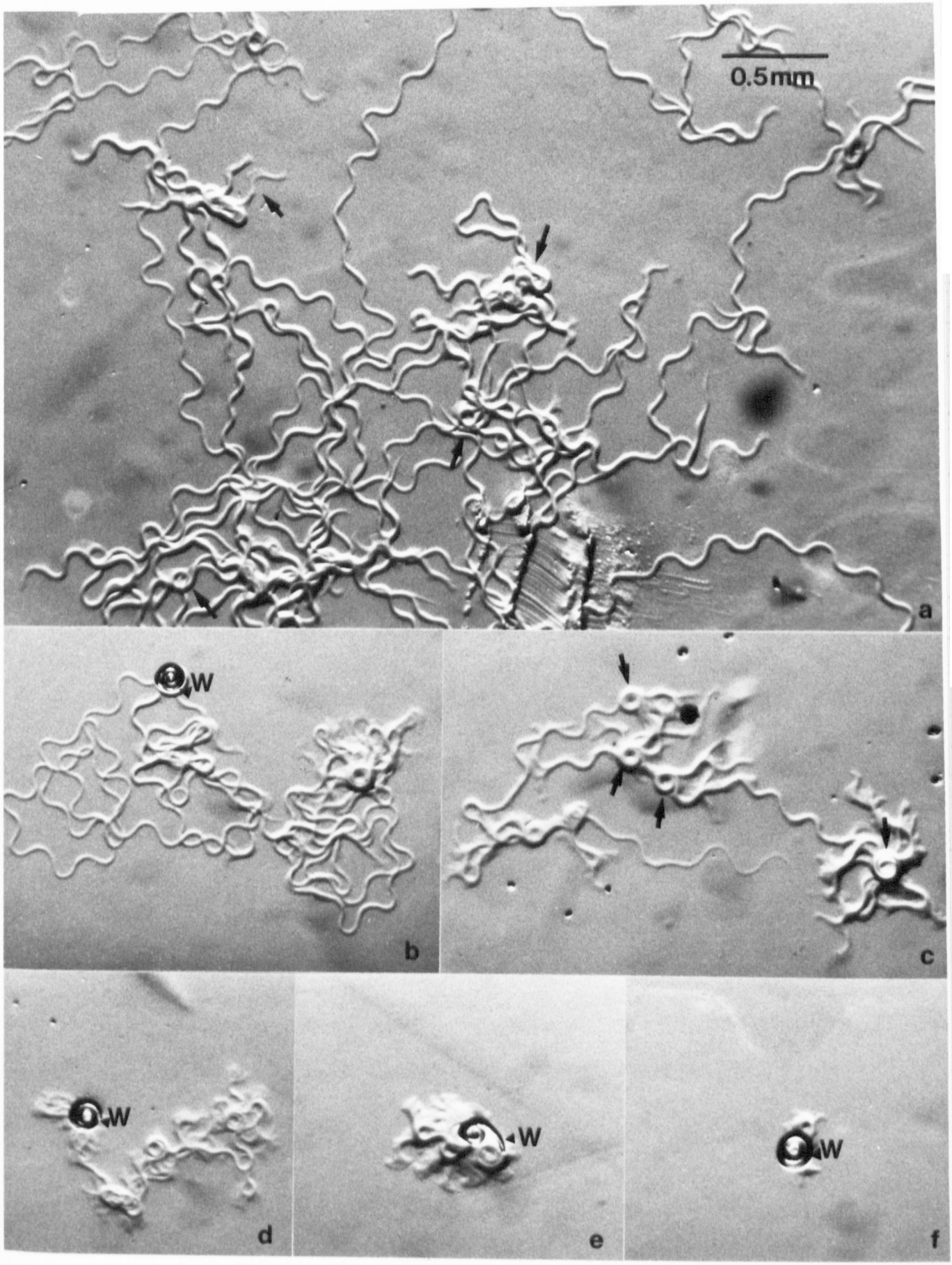


Fig. 7.2

Time in host (hrs)	Moved	NUMBER OF LARVAE (OUT OF 10) THAT				Average distance moved (± SE, mm)
		Left waves	Left waves & wriggles	Left wriggles	Left a pit	
0	10	10	10	0	0	49.9 ± 6.1
6	10	8	2	0	0	21.9 ± 6.9
18	9	1	7	1	0	4.3 ± 1.2
24	10	0	6	3	1	1.8 ± 0.4
48	8	1	2	2	3	1.5 ± 0.5
72	9	0	1	1	7	< 1
96	0	0	0	0	2	< 1

Table 7.1: The ability of third-stage larvae of Nematospiroides dubius to move over agar at 26 - 30°C in 150 minutes. Note that the 10 infective-stages left wave-like patterns and moved over an average distance of nearly 50 mm. Few larvae exposed to the host for more than 24 hours left wave-like patterns or moved more than 1 mm. ± SE, standard error.

it was not possible to quantify these reversals because, more often than not, several occurred in one spot (Fig. 7.2a).

In contrast, after N. dubius had been in a host for 18 hours, movement appeared to be restricted (Fig. 7.2). Not only did these larvae moved over less distance than infective-stages, but their tracks contained more reversals and wriggles (Figs 7.2b and 7.2c). And after being in a host for about 2 days, sinusoidal tracks were rarely observed (Figs 7.2d and 7.2e, Table 7.1). Most of these larvae left wriggles in the agar, hardly moving at all. In some instances, only a pit was visible around the coiled worm to show that it had been active (Fig. 7.2f, Table 7.1).

Although most larvae did not move using sinusoidal waves after being in a host for more than 2 days (Table 7.1), there were a few exceptions. The wavelength and amplitude of wave patterns left by post-infective larvae were significantly different, usually shorter, from those made by infective worms but no trend with age was observed ($P < .01$, see Appendix 4). The mean wavelength of tracks left by infective larvae was 0.21 $\pm$ 0.004 mm ($\pm$ standard error) and the mean amplitude of the wave pattern was 0.11 $\pm$ 0.003 mm ($\pm$ standard error). There was a significant interaction between wavelength and amplitude of the waves ($P < .01$, see Appendix 4).

Observations of third-stage larvae of N. dubius in saline suggested that, while infective larvae could maintain sinusoidal waves along their length, most larvae had lost this ability after 24 hours in a host. When moving on the bottom of a watchglass, late third-stage larvae used coiling-uncoiling movements, rather than undulatory waves. And, unlike infective-stages, late post-infective larvae could not swim through fluid (Fig. 7.3). Instead of maintaining a series of waves along the body, in late third-stage N. dubius the front half of the worm

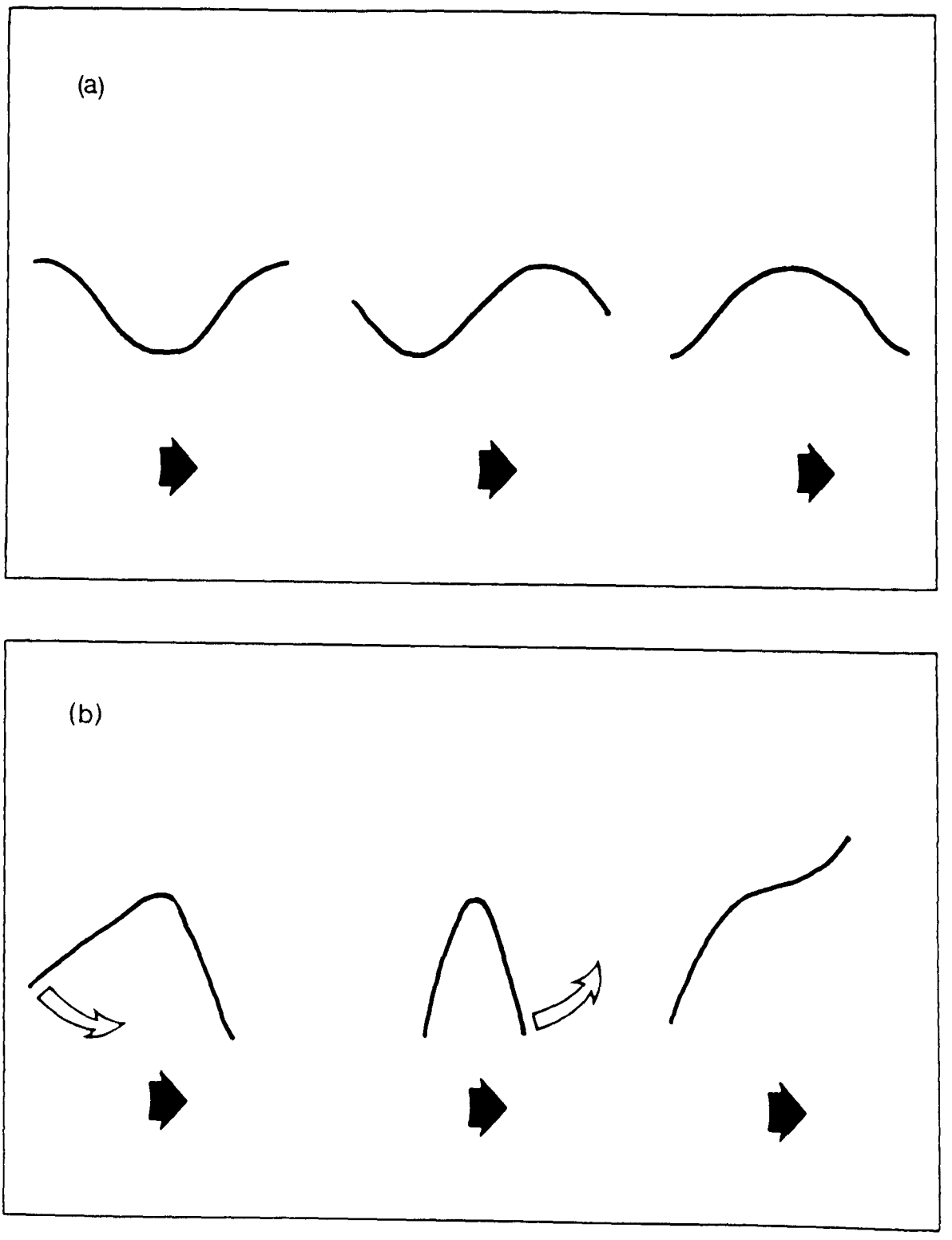


Fig. 7.3: Diagram showing the type of movement exhibited by third-stage larvae of Nematospiroides dubius swimming in saline. Sinusoidal wave-like motion typical of infective stages is shown (a). Third-stage larvae removed from a host 24 hours after infection, have lost the ability to make wave-like movements (b). Instead, the anterior end of the worm bends laterally, usually to the same side and the rear moves up to almost touch it.

bent laterally through more than 45° and then the rear of the worm bent laterally to almost touch the front end (Fig. 7.3).

7.3.2 An ultrastructural study of third-stage larvae prior to the third moult

Apart from these morphological and behavioural changes, the developing third-stage larvae of N. dubius also underwent ultrastructural alterations.

Micrographs taken at low magnification confirmed that larvae increased in diameter as they developed within a host (see Section 7.3.1, Figs 7.4 to 7.7). The diameter of some late third-stage larvae was almost double that of infective-stages. And as larvae increased in diameter, the cuticle became thinner (Table 7.2; Figs 7.4 to 7.7). The ultrastructural changes which took place in the third-stage cuticle will be discussed later.

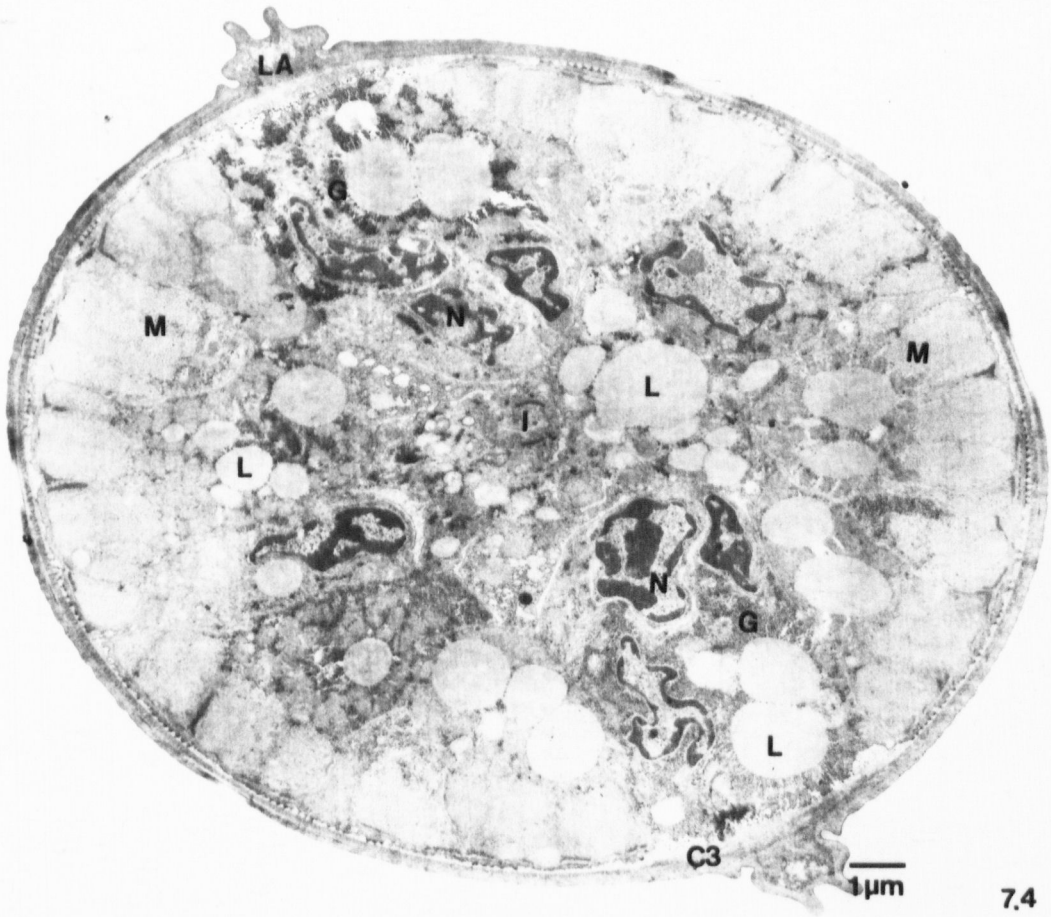
The body wall appeared to be further weakened by a decrease in the amount of muscle fibre (compare Figs 7.4 to 7.7; Table 7.2). At the time third-stage larvae had begun to synthesize a fourth cuticle (Fig. 7.7), the actual width of the muscle fibres viewed in transverse section had decreased by 2 to 3 times.

Other changes apparent within larvae after infection illustrated that the larvae were actively feeding and growing. Within 24 hours of infection, for example, the stores of lipid and glycogen, so abundant in the infective-stage, appeared greatly reduced (compare Figs 7.4 and 7.5). In addition, the appearance of the chromatin present in the prominent nuclei was suggestive of synthetic activity (compare Figs 7.4 and 7.7). Moreover the intestine, which in the non-feeding infective larvae could hardly be identified, was easily recognized. And after

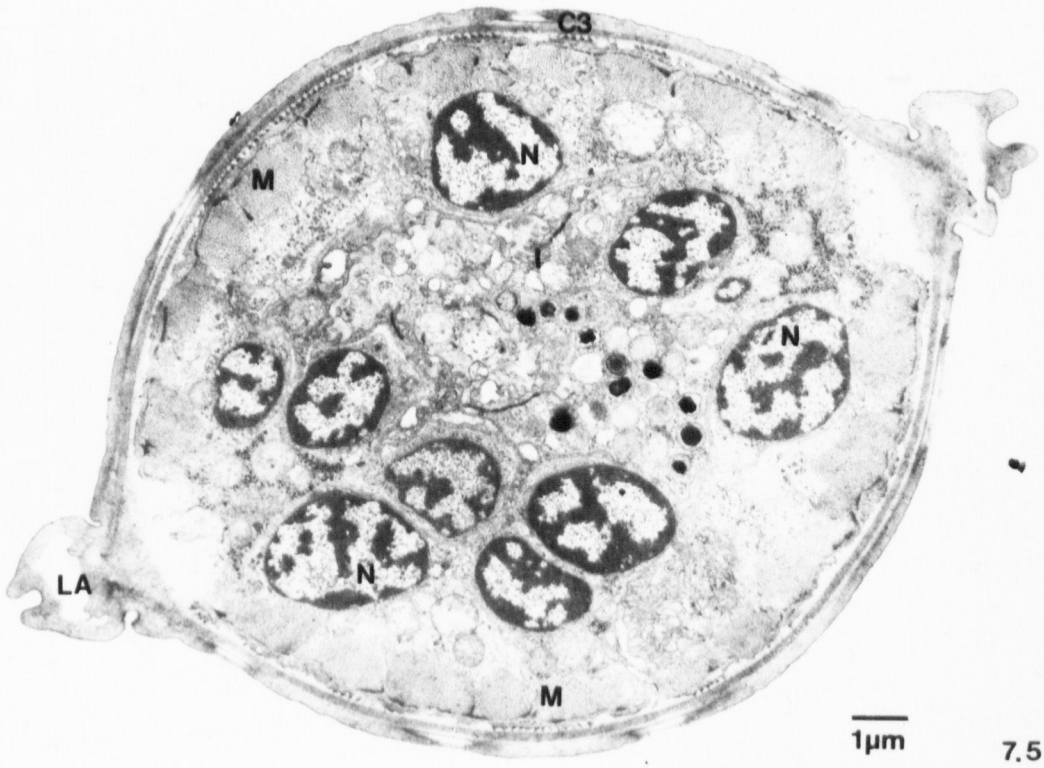
Figs 7.4 to 7.7: Transverse sections of third-stage larvae of Nematospiroides dubius before, and at varying times after infection. Sections were cut from just posterior to the base of the pharynx, except in Fig. 7.7 where the pharynx can be seen. All to the same magnification.

Fig. 7.4: An infective larva which was artificially exsheathed. Note the distinctive lipid (L) and glycogen (G) deposits. Nuclei (N) contain condensed chromatin and the muscle fibres (M) are well developed. Lateral alae (LA) of the cuticle (C3) are prominent, while the intestine (I) is hard to discern.

Fig. 7.5: A larva recovered from the host 24 hours after infection. The appearance of the chromatin within the prominent nuclei (N) is indicative of synthetic activity. Lipid and glycogen has diminished, while small portions of the intestine (I) can be seen (compare with Fig. 7.4). Muscle (M) fibres are well developed and the lateral alae (LA) of the cuticle (C3) are prominent.

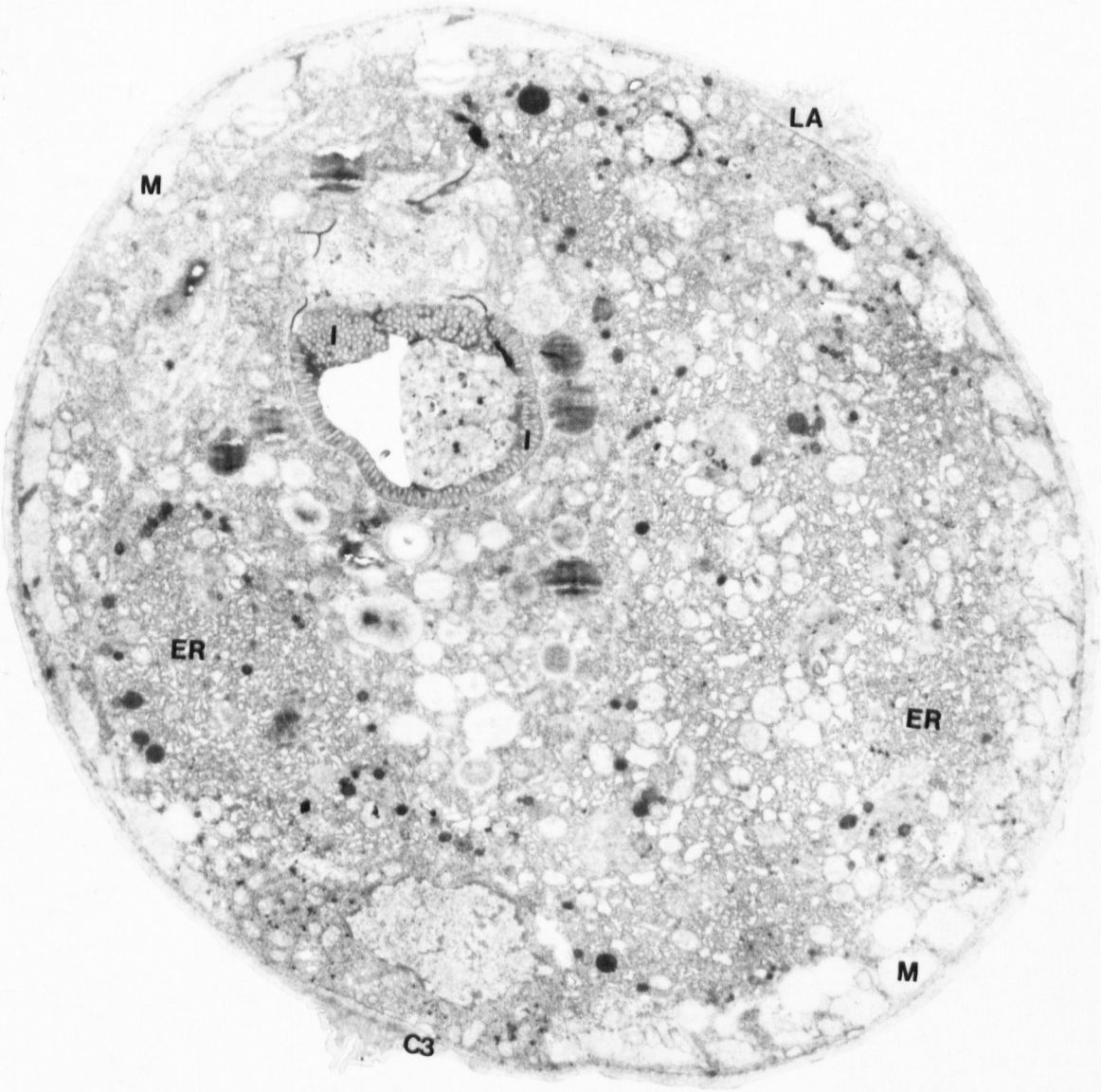


7.4



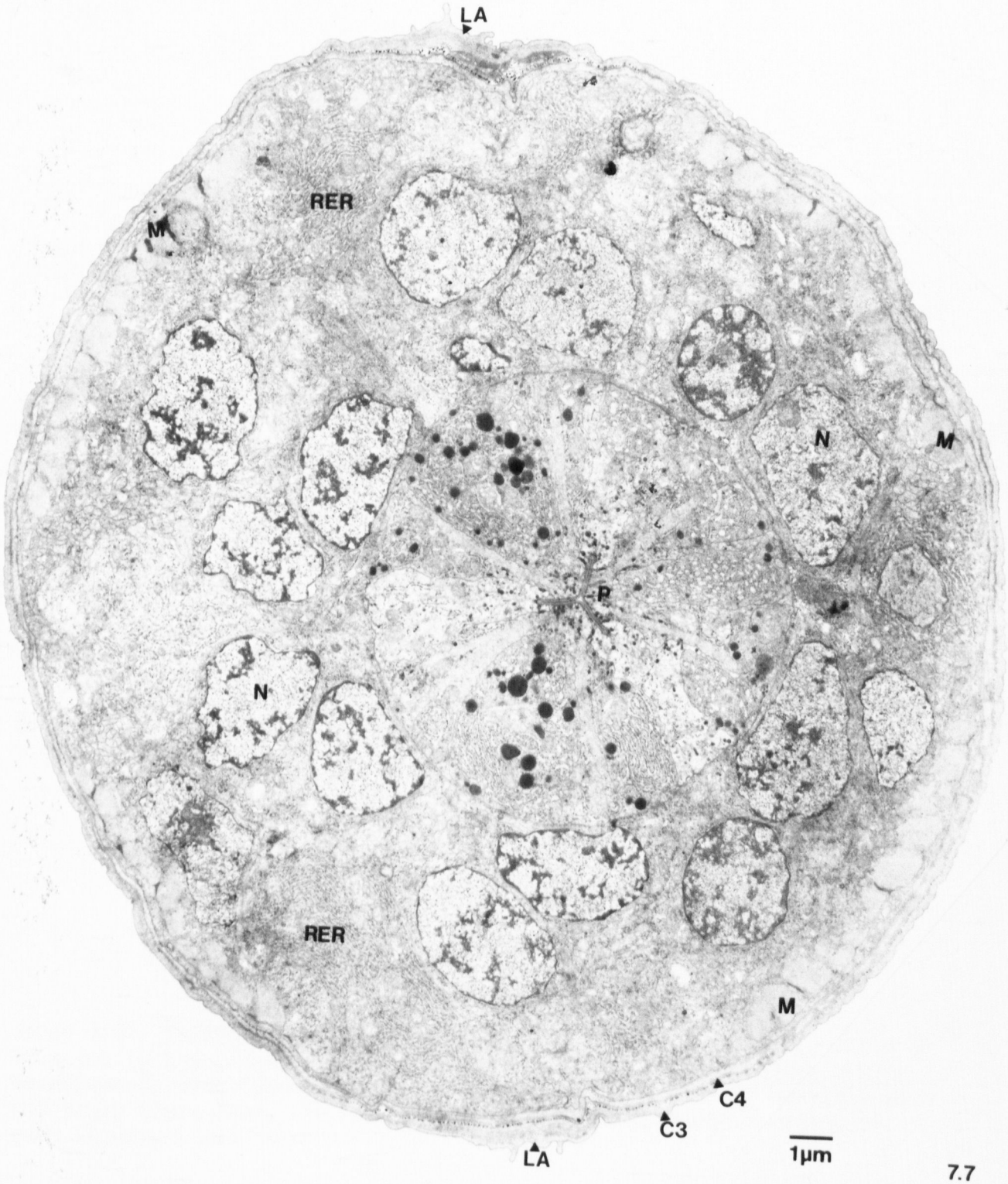
7.5

Fig. 7.6: A larva prior to the formation of the fourth-stage cuticle showing that the worm has increased in girth (compare with Fig. 7.4). Lateral alae (LA) have changed in shape and the cuticle (C3) appears thinner. The intestine (I) is half-filled with cells, presumably of host origin. Muscle fibres (M) occupy a small portion of the worm and the endoplasmic reticulum (ER) is extensive.



7.6

Fig. 7.7: Transverse section through the posterior pharynx (P) of a late third-stage larva. Lateral alae (LA) are much reduced and the new cuticle (C4) is evident beneath the old one (C3). Nuclei (N) are prominent and rough endoplasmic reticulum (RER) is present in the lateral epidermal chords. The area occupied by muscles (M) is reduced (compare with Figs 7.4 and 7.5).



7.7

Stage of development	Width of muscle (nm)	Ratio of muscle to worm diameter	Width of C3 (nm)	Ratio of C3 to worm diameter
Infective stage	1544 - 2049	1 : 10	280 - 349	1 : 45
Prior to apolysis	996 - 1321	1 : 14	335 - 421	1 : 46
Early C4 formation	573 - 749	1 : 31	263 - 280	1 : 77
Late C4 formation	525 - 914	1 : 38	131 - 263	1 : 139

Table 7.2: Thickness of the third-stage cuticle (C3) and muscle fibre in relation to the diameter of developing larvae of Nematospiroides dubius. Measurements were taken from 5 micrographs and because some sections were not truly transverse, the mean of the thinnest and thickest region of each structure was recorded.

2 to 3 days in the host, the lumen of the intestine contained many cells, presumably of host origin (Fig. 7.6).

Some of the changes in the developing third-stage larvae appeared to be associated with the formation of the fourth-stage cuticle which usually took place between 72 and 83 hours after infection, although the timing varied amongst individuals. The four epidermal chords enlarged to occupy a substantial portion of the nematode prior to cuticle formation and before the new cuticle was complete, the amount of rough endoplasmic reticulum in the chords had increased markedly (compare Figs 7.4 and 7.5 with Figs 7.6 and 7.7).

As already mentioned, the third-stage cuticle underwent a number of ultrastructural changes prior to apolysis and the formation of the fourth cuticle (Table 7.3; Figs 7.8 to 7.15). The cuticle of larvae exposed to a host for 24 hours was similar to that of infective-stage larvae (compare Figs 7.8 and 3.7), but by 48 to 72 hours after infection, the basal zone no longer contained striated material (compare Figs 7.8 and 7.9). Instead the basal zone consisted of a band of amorphous material, which was more electron-dense than the cortical zone. As larvae developed further, the basal zone became less dense and thinner (compare Figs 7.9 to Fig. 7.11). Indeed the basal zone was reduced to about a half of its initial thickness at the time of apolysis. Thus the late third-stage cuticle appeared more diffuse in structure than the cuticle prior to infection. (That the section shown in Fig. 7.9 was a true transverse section, and not an oblique section which might hide the striations, is indicated by the appearance of the muscle fibres).

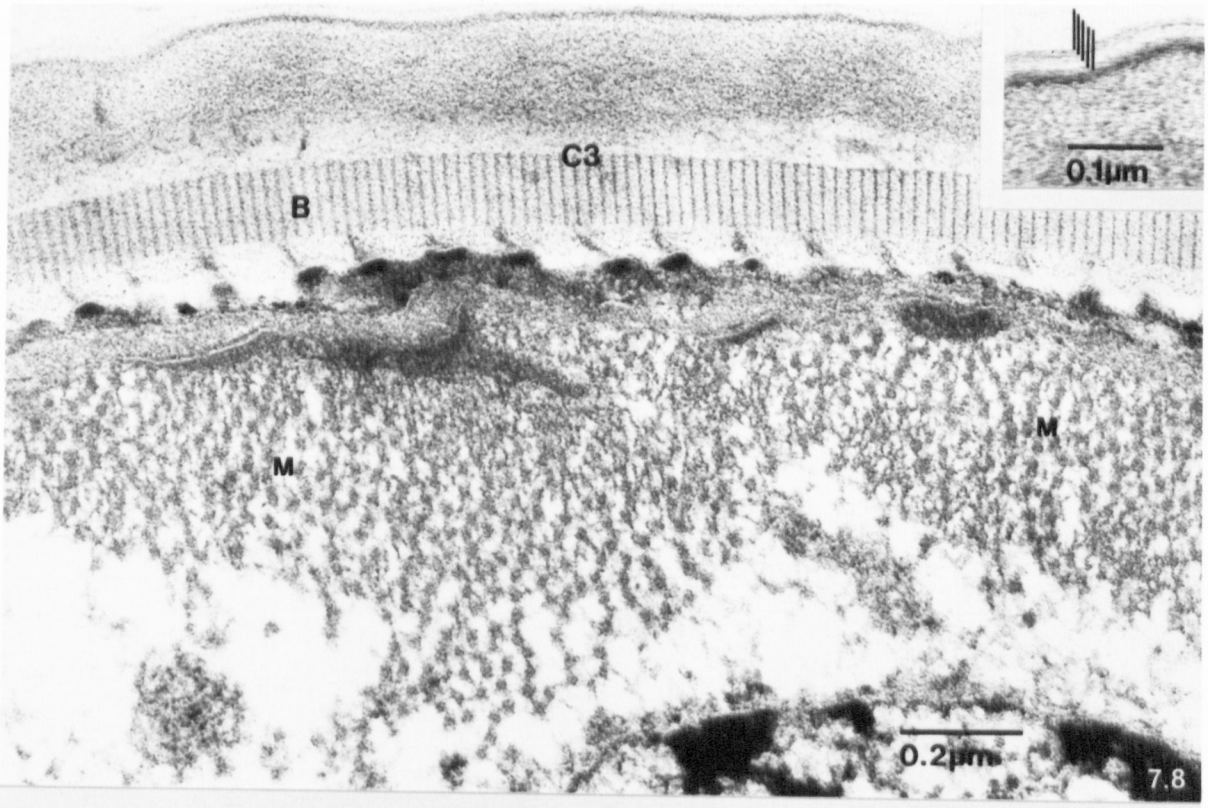
A similar reduction in cuticular thickness was noted in the fourth-stage larvae of N. dubius, just prior to the final moult. Again

Stage of development	Width of cuticle (± SE, nm)	Width of dense band/striations (± SE, nm)	Width of epicuticle (± SE, nm)
Infective stage	312 ± 19.9 to 372 ± 16.9	115 ± 5.3 to 135 ± 8.6	25 ± 0.8 to 29 ± 1.2
Prior to apolysis C3 degenerating	270 ± 17.4 to 340 ± 19	100 ± 12.5 to 114 ± 19.0	21 ± 2.2 to 24 ± 2.6
After apolysis early C4	211 ± 12.9 to 296 ± 22.8	70 ± 2.6 to 75 ± 2.8	12 ± 0.3 to 13 ± 0.5
Late C4 formation	200 ± 11.6 to 310 ± 22.4	55 ± 3.9 to 75 ± 4.6	12 ± 0.3 to 14 ± 0.3

Table 7.3: Changes in the thickness of the third-stage cuticle of Nematospiroides dubius prior to the third moult. ± SE, standard error.

Fig. 7.8: The cuticle (C3) of a larva of Nematospiroides dubius removed from the host 24 hours after infection. The epicuticle is still multilaminate (see inset) and the basal zone (B) contains striated material. Muscle fibres (M) appear in disarray. The larva was labelled with cationized ferritin but none adhered to its surface.

Fig. 7.9: The cuticle (C3) of a larva of Nematospiroides dubius removed from the host about 48 hours after infection and labelled with cationized ferritin (CF). The epicuticle is trilaminate but in some regions a third electron dense lines is present in the outer cortical zone (circled). Faint electron-dense lines are also apparent outside the trilaminate epicuticle (→). No cationized ferritin was seen on the surface of the worm. The basal zone is an electron-dense, amorphous layer (B) and muscle fibres (M) exhibit the pattern characteristic of a transverse section.


C3
B
M
M
0.1µm
0.2µm
7.8

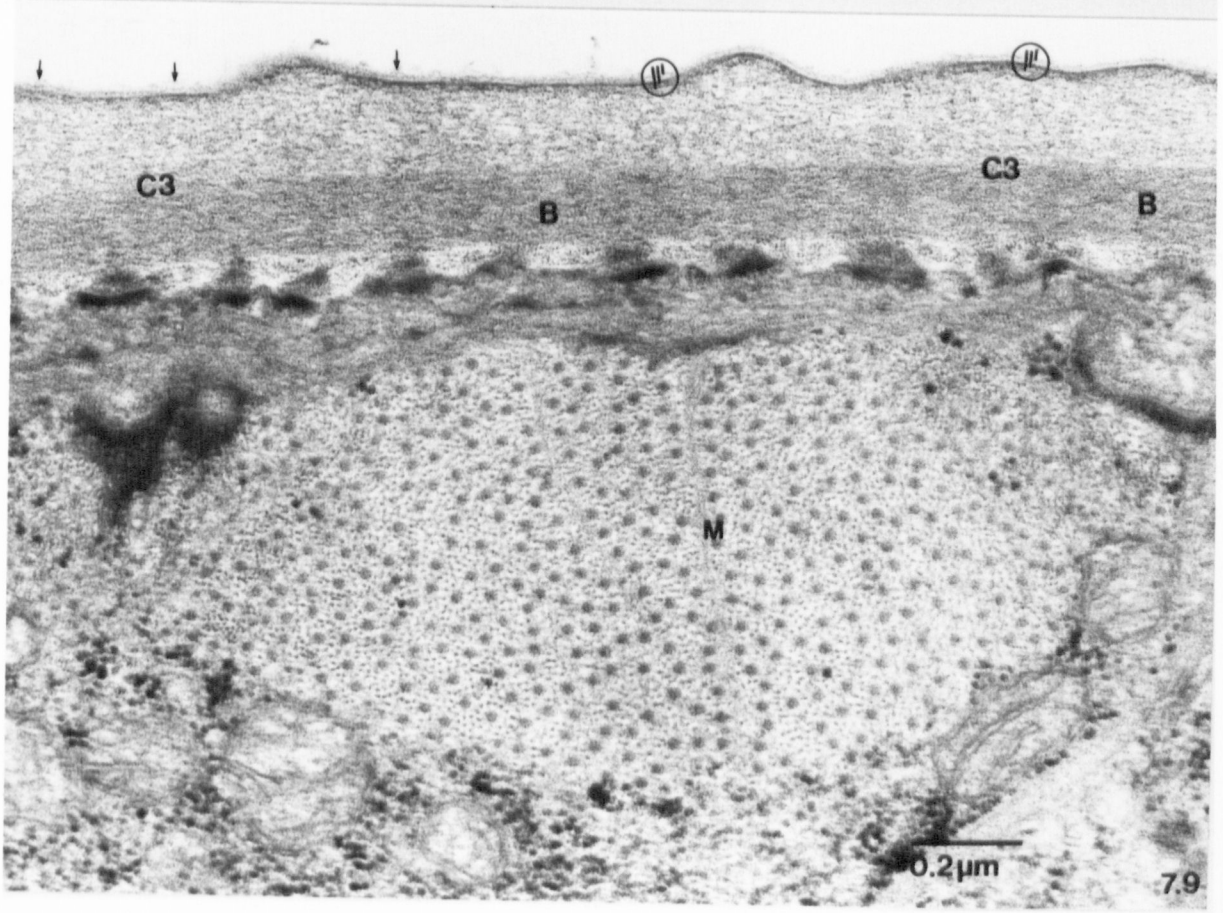
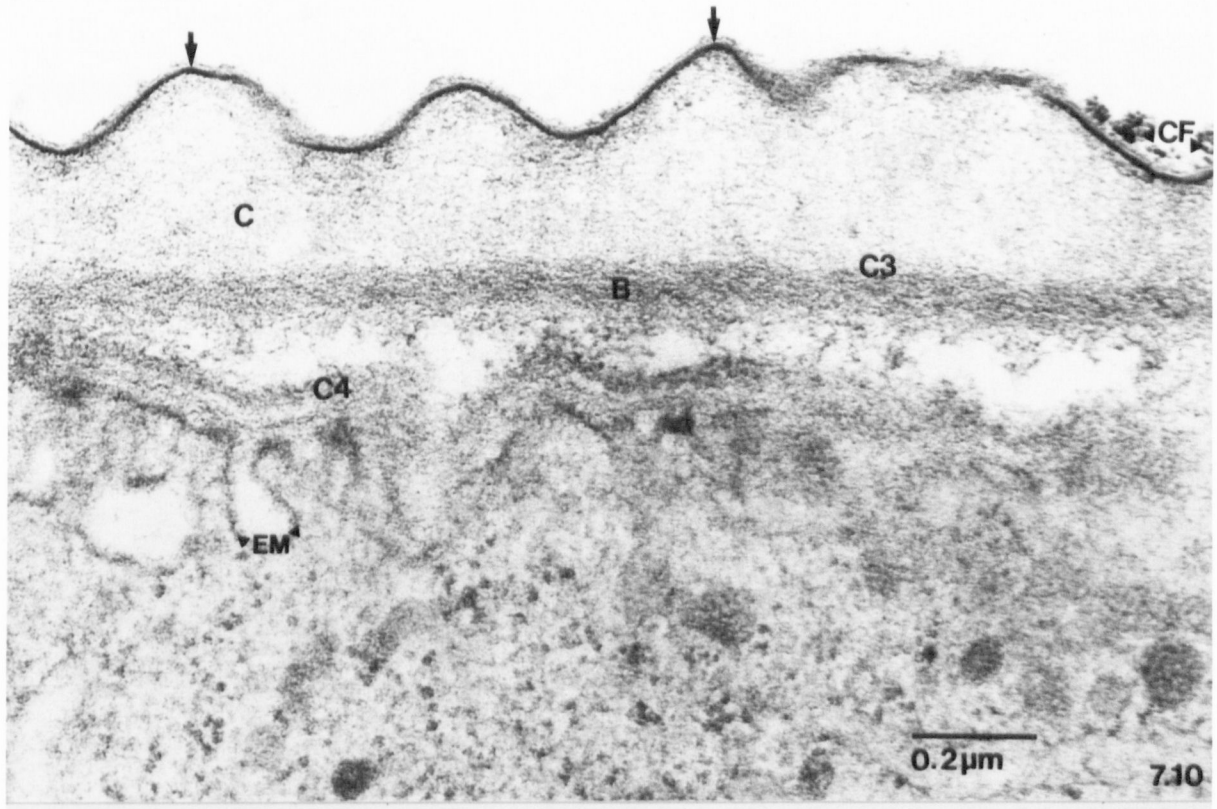
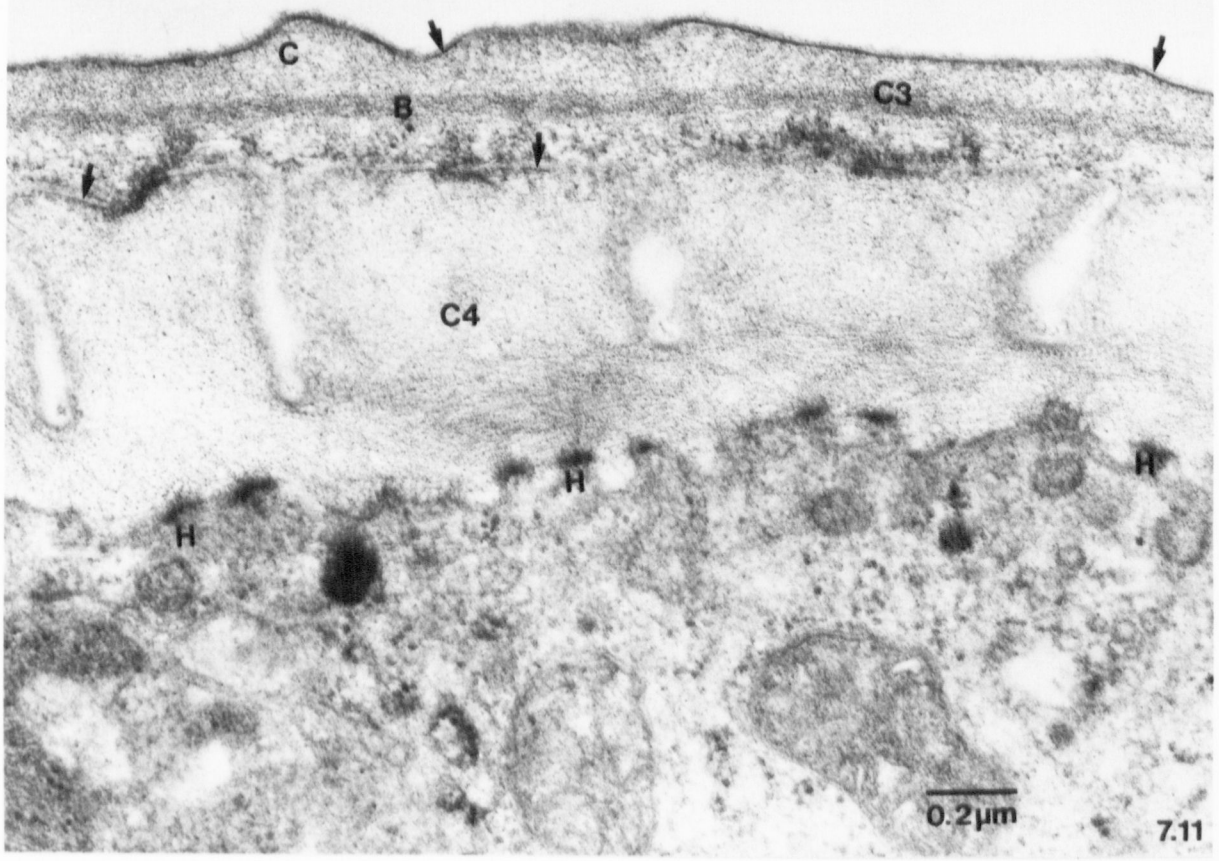

C3
B
C3
B
M
0.2µm
7.9

Fig. 7.10: Transverse section showing early cuticle formation in a larva of Nematospiroides dubius which was labelled with cationized ferritin (CF). The basal zone (B) of the third-stage cuticle (C3) is slightly more electron-dense than the cortical zone (C) and the epicuticle appears trilaminate (→). The early fourth-stage cuticle (C4) is evident above the epidermal membrane (EM).

Fig. 7.11: Transverse section showing late cuticle formation in a larva of Nematospiroides dubius. The new fourth-stage cuticle (C4) is attached to the underlying epidermal membrane at hemidesmosomes (H). The basal zone (B) of the third-stage cuticle (C3) appears narrow and amorphous and the cortical zone (C) is diffuse. Note the trilaminate structure (→) of the epicuticle of both the C3 and C4. The worm was not labelled with cationized ferritin.



7.10



7.11

the innermost fibrous layer became diffuse, and was all but lost before the final moult (compare Figs 7.16 and 7.17 with Fig. 3.12).

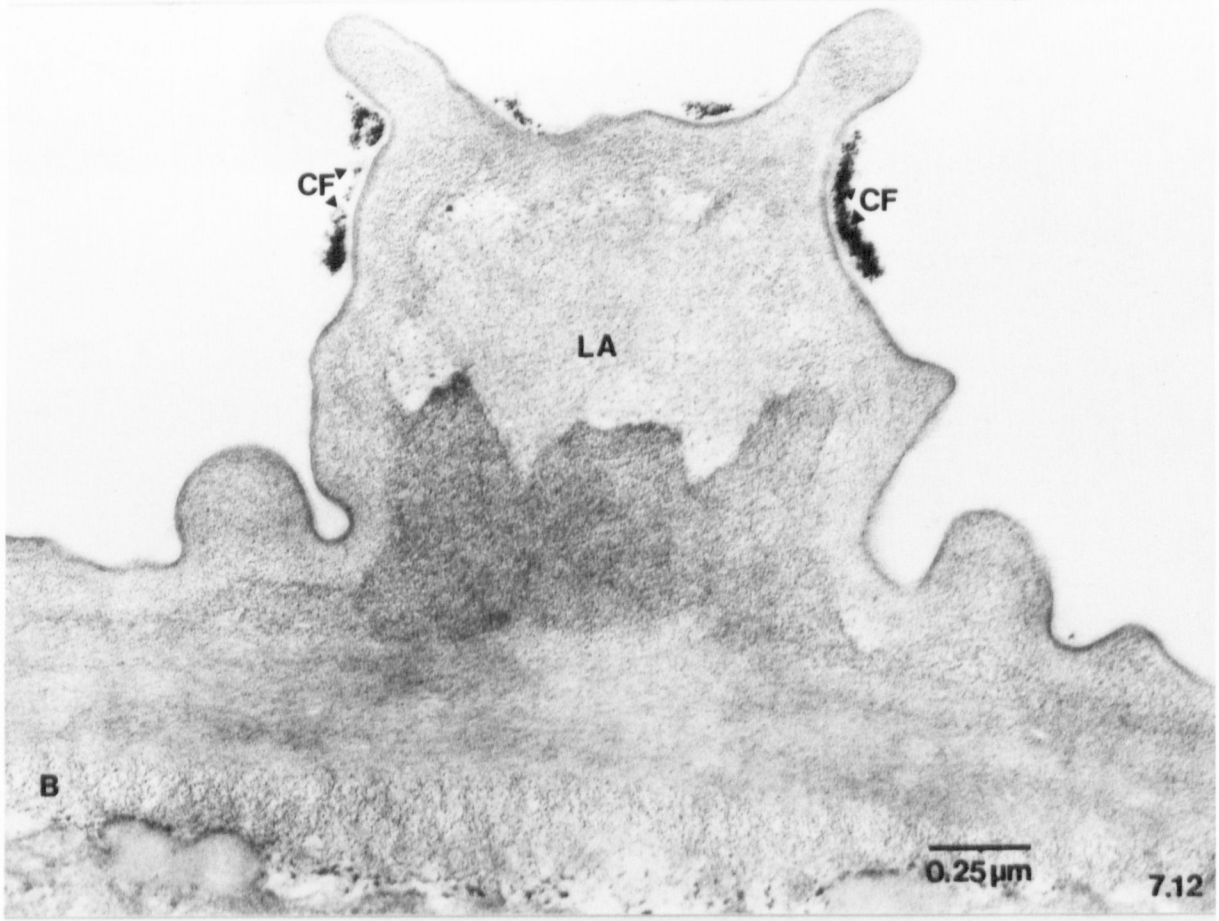
Another outstanding way the third-stage cuticle changed, was in the region of the lateral alae (compare Figs 7.4 to 7.7 and Figs 7.12 to 7.15). The prominent ridges which characterize the lateral alae of the infective cuticle diminished gradually until only the largest could be identified as 2 small knobs.

Less obvious changes were observed in the epicuticle of third-stage larvae of N. dubius during development in the host. The epicuticle maintained its multilaminate structure for at least 24 hours after infection (compare Figs 7.8 and 3.10), but it usually appeared trilaminate just prior to apolysis (Figs 7.9 to 7.11). Occasionally 3 electron-dense lines were observed instead of two (Fig. 7.9). Where the epicuticle was trilaminate, the inner line was always more dense than the outer.

As the epicuticle became trilaminate, further changes were apparent on the surface of larvae. Unlike the smooth surface of the infective cuticle (C3) prior to infection, the surface of the third-stage cuticle after 48 hours in the host was "fluffy" in appearance (compare Figs 3.7 and 3.10 with Figs 7.9 to 7.11). A change in the surface of the cuticle was evidenced by its reaction with cationized ferritin. Infective-stage larvae, and of those exposed to the host for 24 hours, showed no attraction for cationized ferritin (compare Fig. 7.8 and 6.3), but clumps of cationized ferritin bound in some circumscribed regions to worms recovered from a host about 48 hours after infection (Figs 7.9, 7.10 and 7.12). Neither Con A nor WGA were detected on the surface larvae removed from a host 24 or 72 hours after infection.

Fig. 7.12: A lateral ala (LA) of a larva of Nematospiroides dubius prior to apolysis. The prominent ridges of the lateral ala are smaller (compare with Fig. 7.4) and striated material is absent from the basal zone (B). Cationized ferritin (CF) bound to the surface of the worm which was removed from the host 72 hours after infection.

Fig. 7.13: The shape of the lateral ala (LA) of an unlabelled larva of Nematospiroides dubius prior to apolysis differs from that of infective larvae (see Fig. 7.4). The base of the LA appears to have been pulled out. The epicuticle is trilaminate (→).


CF
CF
LA
B
0.25 µm
7.12

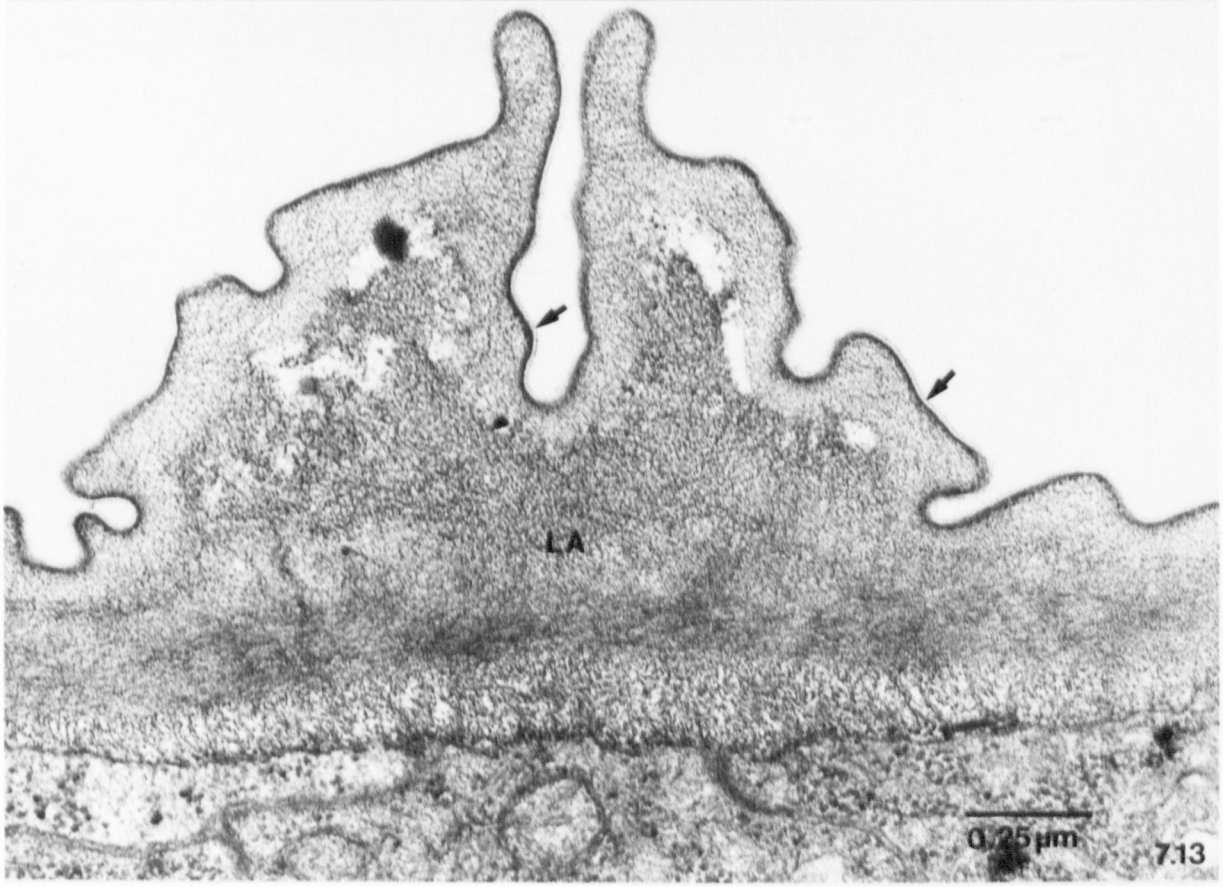
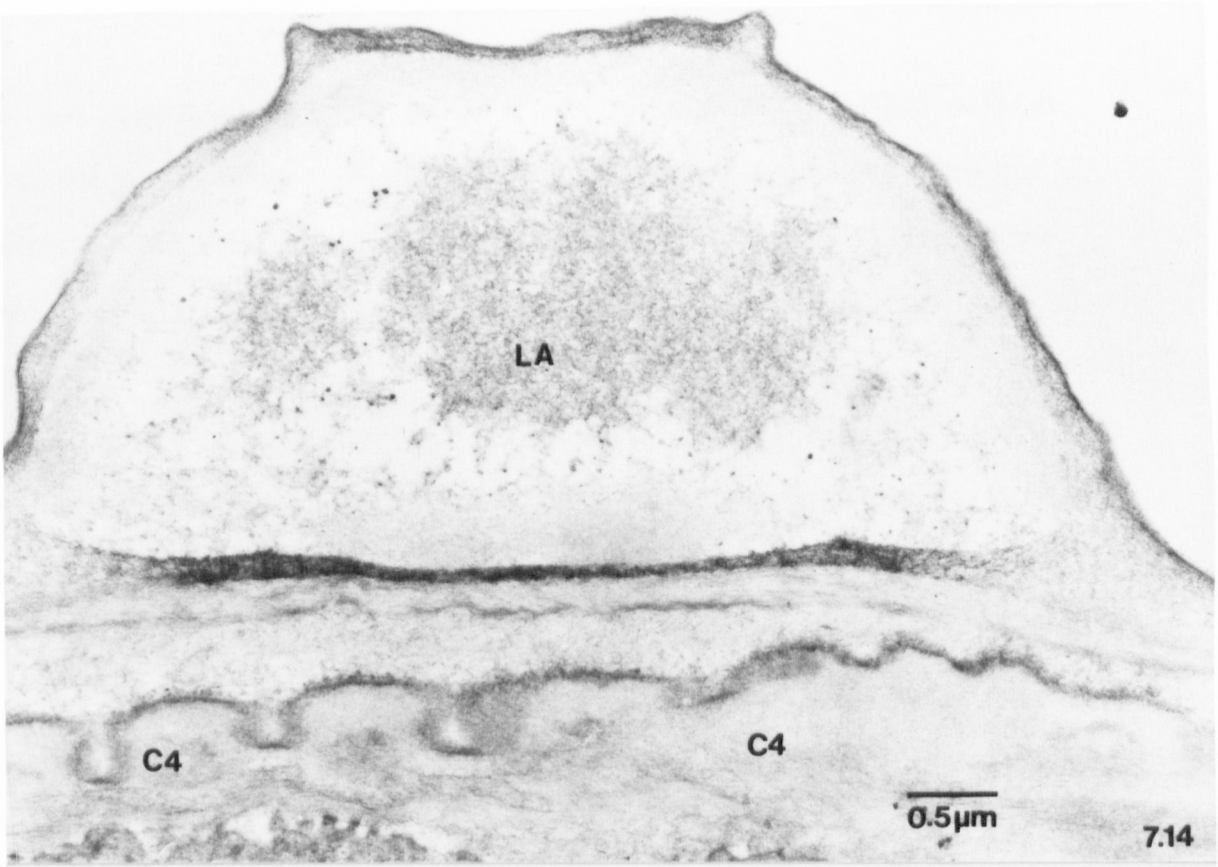
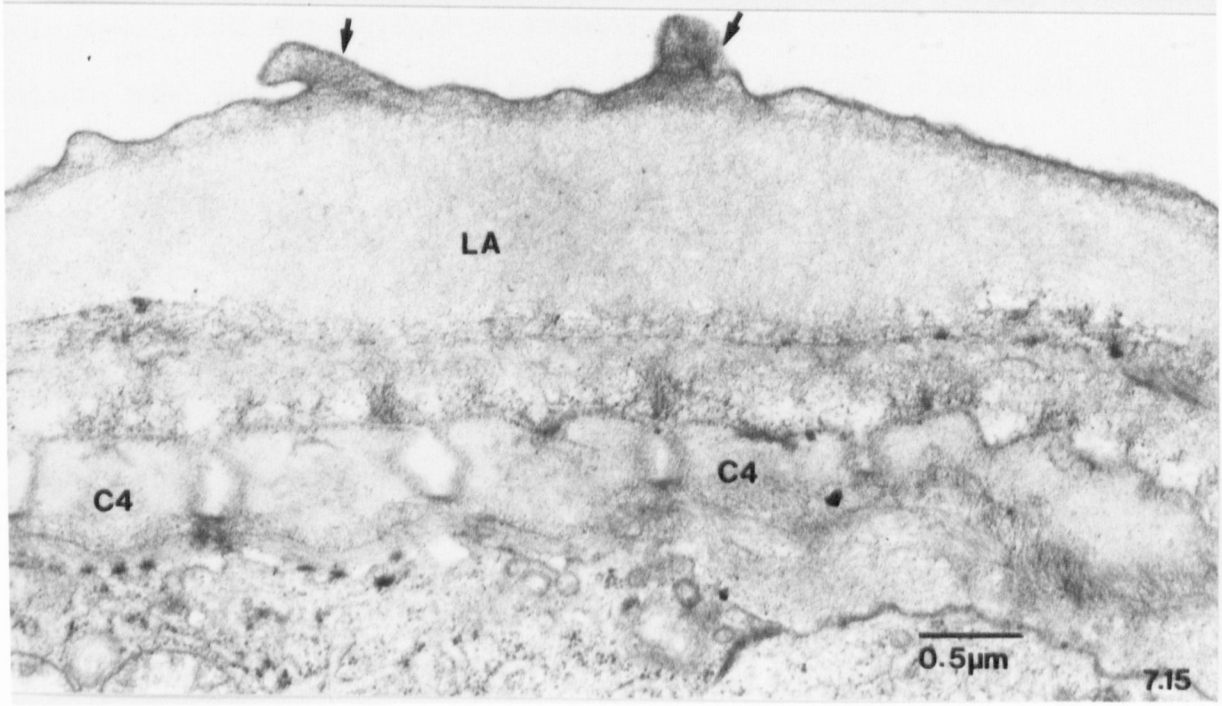

LA
0.25 µm
7.13

Fig. 7.14: The lateral ala (LA) of a late third-stage larva of Nematospiroides dubius lacks the structure found in the infective cuticle (see Fig. 7.4). Note the underlying fourth-stage cuticle (C4).

Fig. 7.15: Apart from 2 knobs (→), the lateral ala (LA) of this late third-stage larva of Nematospiroides dubius is hardly visible. Note the underlying fourth-stage cuticle (C4).



7.14



7.15

The epicuticle of the third-stage cuticle of N. dubius altered during the early stages of parasitism. Comparable changes were not observed in the epicuticle of fourth-stage larvae as they developed within the wall of the host intestine (compare Fig. 7.16 to Figs 3.12 and 3.13).

No single ultrastructural change in third-stage larvae corresponded with alterations in their behavioural patterns (Figs 7.18 to 7.21). Out of 18 larvae examined, 5 left wave-like patterns, 7 left trails of wriggles and 6 remained virtually stationary (see Appendix 5). In all specimens, the cuticle was still attached to the epidermis. Moreover, although the only worm in which striated material was prominent also produced wave-like movements (Fig. 7.18), larvae in which the striated material was less distinct could also move using sinusoidal waves (Fig. 7.19). And, although striated material was usually absent from the cuticle of worms that could wriggle, faint striations were sometimes seen in worms that did not move (Figs 7.20 and 7.21).

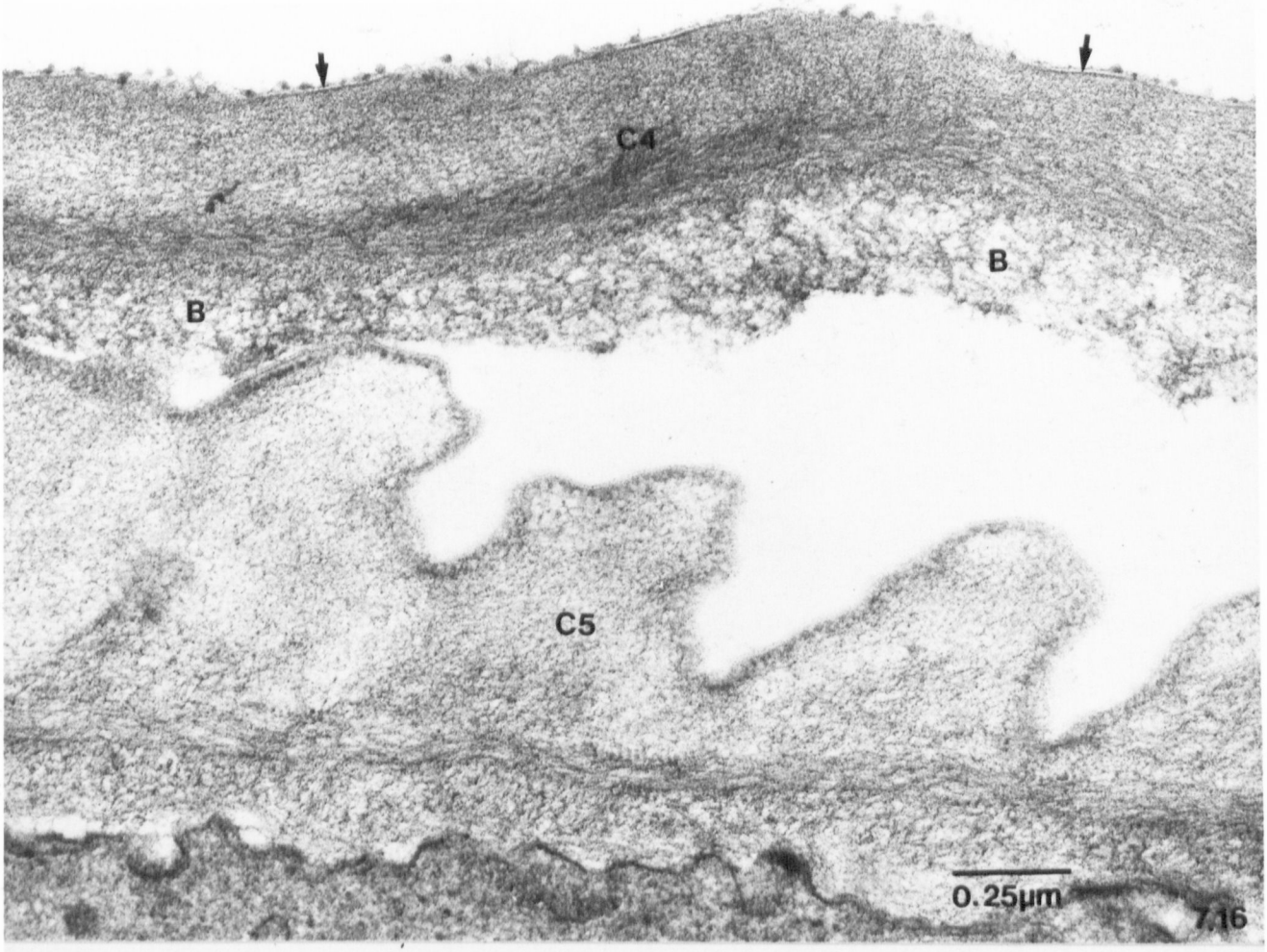
In addition, the appearance of the muscle fibres could not be directly linked with the loss of movement. Muscle within larvae that produced wave-like movements sometimes appeared in disarray, while intact muscle fibres were sometimes seen in worms that moved very little (Figs 7.18 and 7.21).

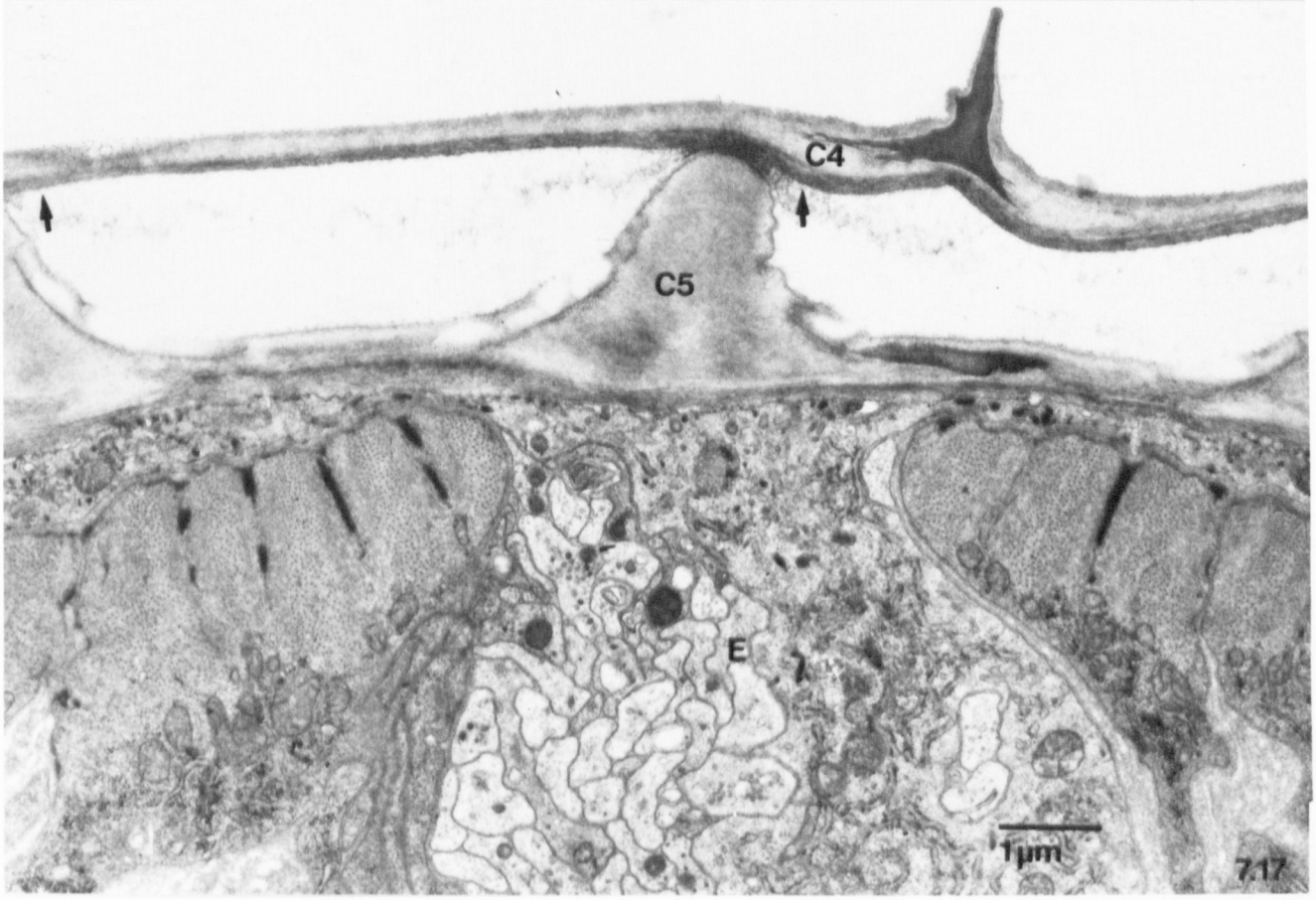
7.4 DISCUSSION

When N. dubius infects a host, the first obvious morphological change is exsheathment which takes place within 5 to 10 minutes (Cypess, Pratt & Van Zandt, 1973; Sommerville & Bailey, 1973). By 14 hours after

Fig. 7.16: The fourth-stage (C4) cuticle of a larva of Nematospiroides dubius prior to the final moult. Note the diffuse inner basal zone (B) of the C4 (compare with Figs 7.17 and 3.12) and the underlying adult cuticle (C5). The trilaminate epicuticle and surface coat (→) of the C4 are distinct.

Fig. 7.17: Transverse section through a lateral epidermal chord (E) of Nematospiroides dubius. The inner basal zone of the fourth-stage cuticle (C4) has broken down (→) above the new adult cuticle (C5).


C4
B
B
C5
0.25µm
7.16


C4
C5
E
1µm
7.17

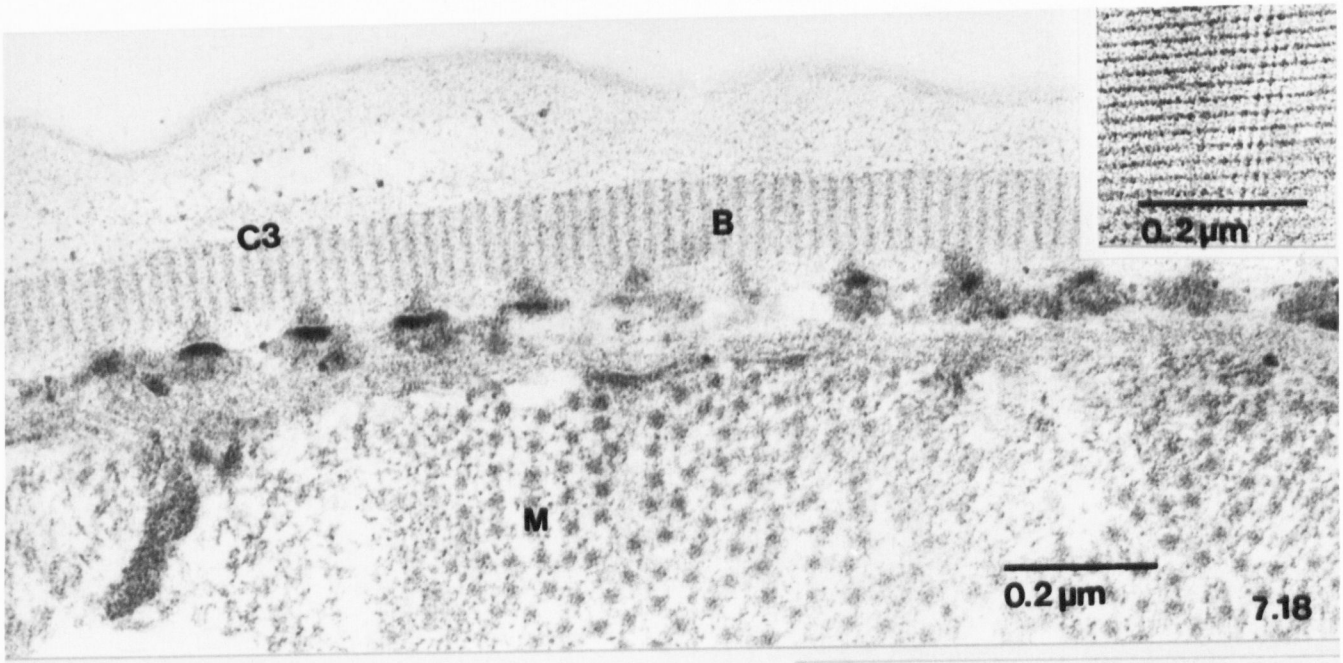
Figs 7.18 to 7.20: The cuticle (C3) of larvae of Nematospiroides dubius removed from the host 54 hours after infection. Larvae were placed on agar plates and left overnight at 37°C prior to fixation.

Fig. 7.18: Cuticle of a larva that left a wave-like pattern in agar. Note the prominent striated material in the basal zone (B) which is also seen in a longitudinal section of the same specimen (see inset). The muscle fibres (M) appear to be disarray.

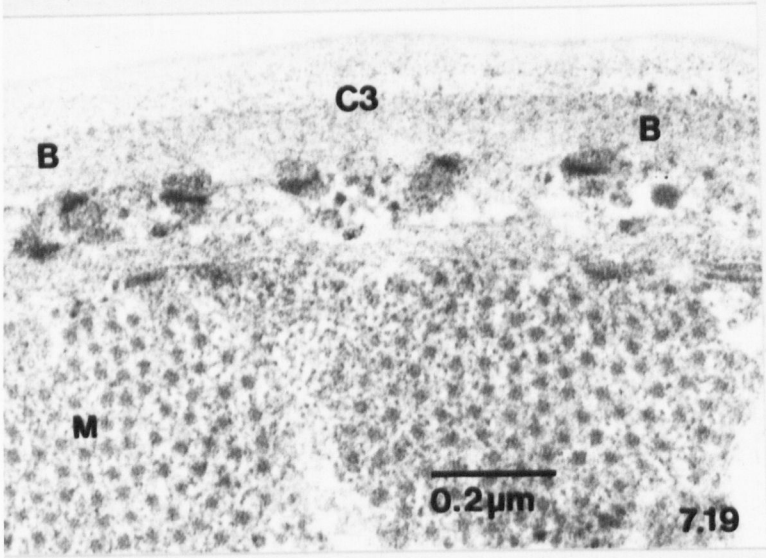
Fig. 7.19: Transverse section of the C3 of a larva that left a wave-like pattern in agar. The material making up the basal zone (B) is not distinctly striated and the muscle fibres (M) are intact.

Fig. 7.20: The cuticle (C3) of a larva which did not move over agar. Faint striations are present in the basal zone (B) and the muscle appears in disarray.

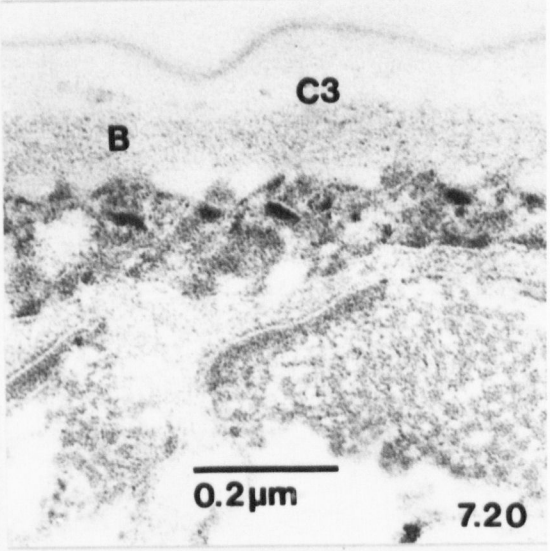
Fig. 7.21: Transverse section through the cuticle of a larva that left wriggles in agar. No striated material is present in the basal zone (B) and the muscle fibres (M) appear largely intact.



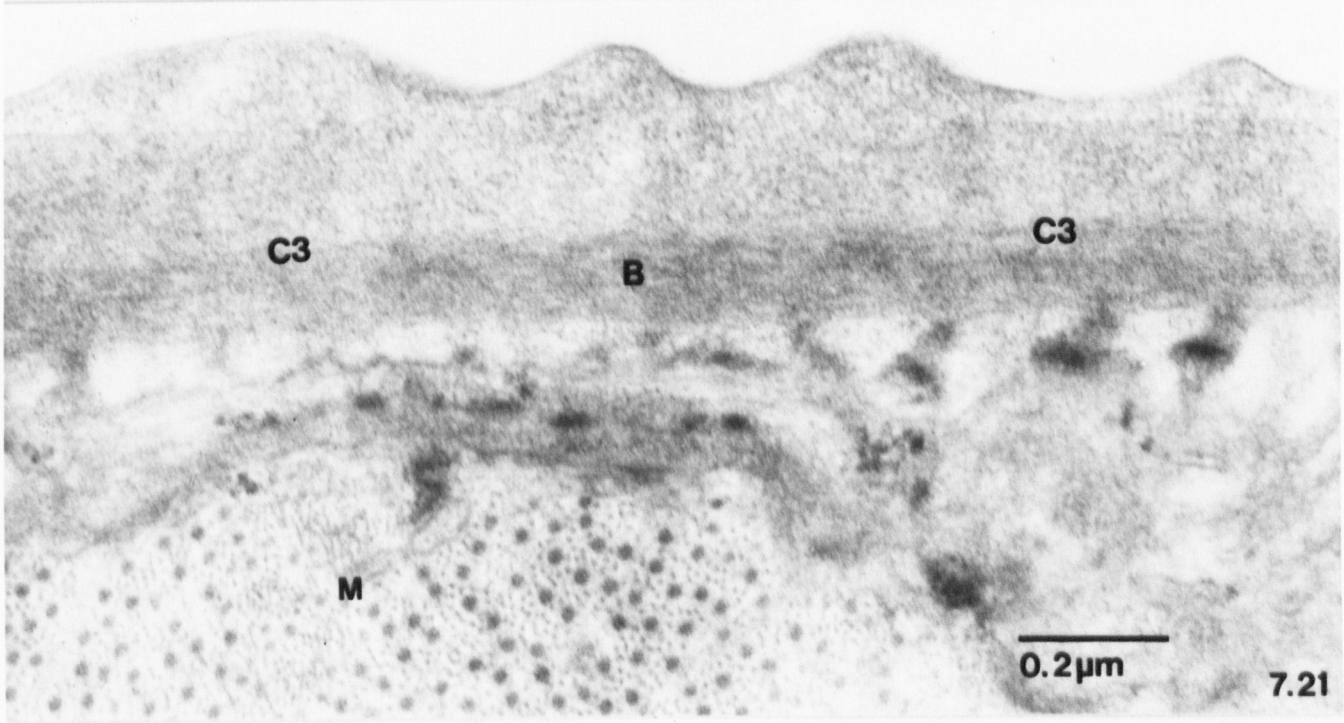
7.18



7.19



7.20



7.21

infection, larvae are capable of ingesting dye *in vitro* (Sommerville, 1969) and presumably can also feed in the host.

The events reviewed in this chapter show that the exsheathed larva then begins to grow and to change the ultrastructure of the third-stage cuticle. Associated with these events are behavioural changes.

7.4.1 Growth

N. dubius underwent a substantial increase in bulk between infection and the third moult. The diameter of larvae, at least in the region behind the base of the pharynx, increased by a factor of nearly two. But larvae did not undergo a great increase in length. After 64 hours in a host, worms were approximately 30% longer than infective-stages, a negligible increase when compared with other species. The length of fourth-stage and adult *A. lumbricoides*, for example, can increase by about 8 times without moulting (O'Grady, 1983; Roberts, 1934; Watson, 1965).

Presumably some of the energy required for this increase in size comes from feeding, but we do not know when the newly established worms start to feed. Sommerville (1969) suggested that feeding may not commence immediately. In the third-stage larvae that I examined, ingesta was identified within the lumen of the intestine of worms three days after infection (Fig. 7.6). If there is a delay in the onset of feeding, a significant source of energy could be reserves of carbohydrate and lipid, both of which were judged to decrease early in infection in this study (Figs 7.4 to 7.7). Similarly, Croll, Matthews and Smith (1975) found that the amount of lipid in larvae of *Ancylostoma tubaeforme* declined over the five days after infection.

The variable nature of the cuticle of nematodes is highlighted by the way in which it grows. While in some species, such as

A. lumbricoides (Watson, 1965), the cuticle grows by acquiring further layers, the cuticle of other nematodes is "stretched" or "plastically deformed" to accommodate the growing worm. Stretching allows a constant amount of cuticular material to spread over a greater area. As third-stage larvae of N. dubius grew, for example, the striated material disappeared from the basal zone of the cuticle which then appeared increasingly diffuse, and the lateral alae appeared to have been "pulled out" by the increasing girth of the worm (Figs 7.12 to 7.15). Similar observations were made on the second-stage larvae of A. agrostis as they developed into dauer forms (Bird & Stynes, 1981).

Of the nematode cuticles that do not increase in thickness, however, not all are stretched during growth. Some form as highly folded structures which allows the organism to grow without new material being added. Larvae of B. pahangi, for example, increased in length without increasing in surface area because the highly folded cuticle is pulled out by the growing worm (Howells & Blainey, 1983; Aoki, Vincent, Ash & Katamine, 1980). The fourth-stage cuticle of N. dubius also formed as a highly folded structure and accordingly, did not alter to the same extent as the third-stage cuticle during moulting (Figs 7.16 and 7.17). The inner fibrous layers of the basal zone broke down, but the longitudinal ridges remained intact. Indeed, this might be a comon device in nematodes. Certainly it is recognized in insects, such as the tobacco hornworm, which can grow continuously because the folded epicuticle allows for expansion (Wolfgang & Riddiford, 1981).

Thus with the resumption of development in N. dubius following infection, the genes governing the formation of the first parasitic stage are expressed, and the worms begin to feed and grow. As the larvae increase in diameter, the lateral alae disappear but no

appreciable increase in length occurs until after the third moult, presumably because the third-stage cuticle is incapable of growth.

7.4.2 Changes in the epicuticle

The epicuticle of the third-stage cuticle underwent substantial alteration about 48 hours after infection. The outer 2 electron-dense lines, as well as the innermost line making up the epicuticle disappeared (Fig. 7.9). Associated with this change, the smooth surface of the epicuticle became "fluffy" in appearance. Moreover, cationized ferritin, which did not bind to the surface of the infective cuticle, adhered to the late third-stage cuticle in circumscribed regions.

The origin of the fine filaments and negatively charged molecules on the surface of the post-infective cuticle (C3) is unknown. Some of the filamentous material probably represents fragments remaining from the outer 2 layers which are lost (Fig. 7.9). But because a fine surface coat is present up to the time of the third moult (Fig. 7.11), it seems likely that some of these filaments are integral components of the newly exposed surface. Alternatively, it is possible that substances from the host adhere to the larval surface altering its appearance and composition.

These changes in the third-stage epicuticle are of particular interest because they occurred before cuticle and epidermis separated at apolysis and are, therefore, likely to be under the control of the worm. The epicuticle apparently altered shortly after the nuclei began synthetic activity (Figs 7.5, 7.6 and 7.13), so the changing surface of *N. dubius* may well be linked with other developmental events.

Similar changes have been observed in the epicuticle of the parasite, *T. spiralis*. Wright (1982) found that the outermost electron-dense line making up the multilaminate epicuticle of

first-stage infective larvae, as it lies encysted in host tissue, was lost between 3 and 6 hours of the worms entering the columnar cells of the host intestine. The origin of the outermost layers of T. spiralis has not been established but in vivo studies using trypsin indicate that their removal is essential for development to resume, and that their removal alters the permeabilty of the cuticle (Berntzen, 1965; Despommier, 1975; 1982). The surface of S. ratti also appears to alter following infection. Prior to infection, labelled antibody complexes were shed from the surface of infective-stage larvae, but no shedding of antibody complexes was observed from third-stage larvae that had been removed from a host (Murrell & Graham, 1983). Alterations to the epicuticle of infective-stage worms may be a widespread phenomenon associated with the resumption of development.

The properties which are unique to the surface of the third-stage larva of N. dubius might be related to its transition to parasitism. Of the different stages of N. dubius, the third-stage larva is the only one in which the epicuticle is multilaminate, and does not exhibit a net negative charge (see Chapter 6). Moreover, when the third-stage epicuticle alters to form a trilaminate structure after infection, it still differs from that of the other four stages. Although all are trilaminate, when the late third-stage cuticle is labelled with cationized ferritin, the distribution of the ferritin is not as even, nor as abundant, as that found on other stages (see Chapter 6). Instead clumps of cationized ferritin molecules bound to some regions of the late third-stage epicuticle (compare Figs 7.10 and 7.12 with Figs 6.1 to 6.6).

Because changes occur in the ultrastructure of the epicuticle of N. dubius following infection, one must assume that the epicuticle of the free-living infective-stage larva, which lies beneath the

second-stage sheath, is unsuited for life within a host. The loss of the outer 2 layers might then ensure the survival of the larvae when it resumes development in the host by, for example, altering the permeabilty of the cuticle.

Alternatively, the outer layers of the epicuticle may be lost in response to contact with the host. Some metazoan parasites are thought to shed and replace components of their surface to prevent host cells from adhering (Hanna, 1980; Smithers, McClaren & Ramalho-Pinto, 1977). It would be of great interest to follow the development of N. dubius in vitro to discover whether the same changes occur in the epicuticle when third-stage larvae are made to exsheath using carbon dioxide instead of a host.

The loss of the outer layers of the epicuticle of N. dubius following infection is particularly interesting in view of studies which suggest that the nematode surface components can be shed (Grove & Blair, 1981; Murrell & Graham, 1983; Philipp et al., 1980b; Smith et al., 1981; Vetter & Klaver-Wesserling, 1978).

Thus, when N. dubius infects a host a number of changes are set in motion. Not only do worms begin to feed and grow in the new environment, but the composition of the cuticle and its surface also alters. And although evidence is lacking, it can be speculated that these changes are under the control of the parasite and a necessary part of the development of parasitism.

7.4.3 Changes in behaviour

Contrary to a suggestion of Croll (1975), nematode larvae can undergo basic behavioural changes without moulting. The behaviour of third-stage larvae of N. dubius altered, prior to the third moult, following the invasion of a host.

Third-stage larvae of N. dubius, which had been exposed to a host for more than 18 hours, no longer used characteristic sinusoidal movements. Instead the movement of these worms was restricted to a series of wriggles on agar (Fig. 7.2), and in fluid they used coiling-uncoiling movements and lateral bending (Fig. 7.3). Coiling movements and lateral bending have a common structural basis because, unlike undulatory movements, both types of behaviour involve contraction of muscles down only one side of the body (Fig. 7.22; Croll 1970).

Comparable changes in behaviour have been recorded for T. spiralis. Once first-stage larvae of T. spiralis entered the small intestine of the host, their movements switched from coiling-uncoiling to being wave-like (Despommier, 1982).

Having established that the behaviour of nematodes can alter without a new cuticle being formed, one is led to ask what causes these changes. Does the type of movement exhibited by a nematode alter in response to an environmental stimulus which acts directly on the worm via the nervous and/or neurosecretory system, or are these behavioural changes the result of other events that occur within the organism due to the resumption of development ?

Free-living infective larvae of N. dubius, for example, may need to move some distance to reach a place where they are likely to be ingested by a host. Clearly this type of movement is not required once inside the mouse. About one-third of worms do migrate temporarily deep into the gastric mucosa for an unknown reason, but the majority move straight into the wall of the small intestine. Are the changes seen in the ability of N. dubius to move a reflection of the onset of a "stationary phase" in which the animal lies coiled in the gut wall ? In this context it is interesting to note that the first-stage larvae of

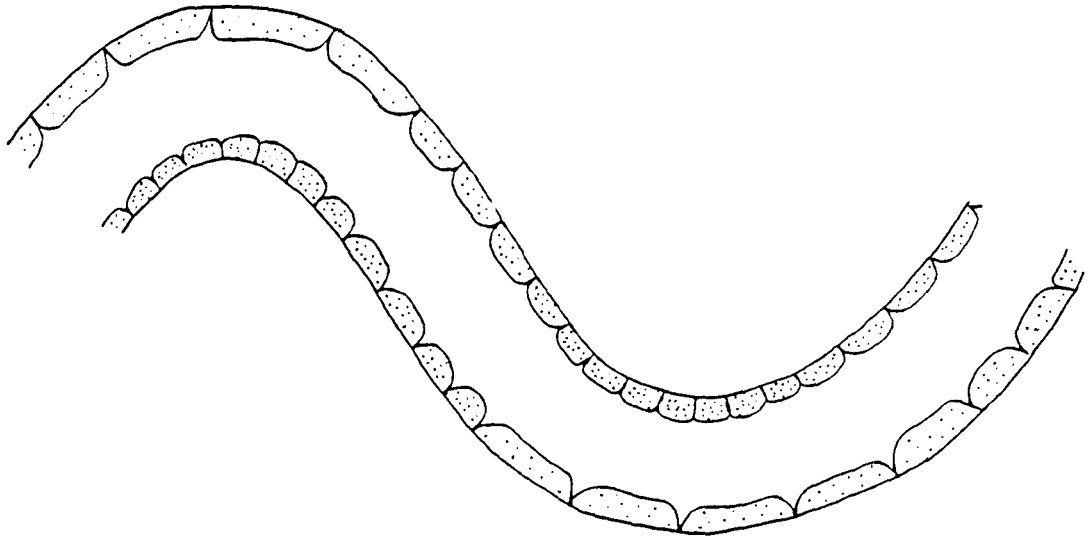


Fig. 7.22(a): Diagram depicting the state of contraction of dorso-lateral and ventro-lateral muscles of a nematode showing a static wave pattern (after Crofton, 1966). Note the ventral and dorsal pairs of muscles are in opposite phases of contraction or relaxation at any part of the body.

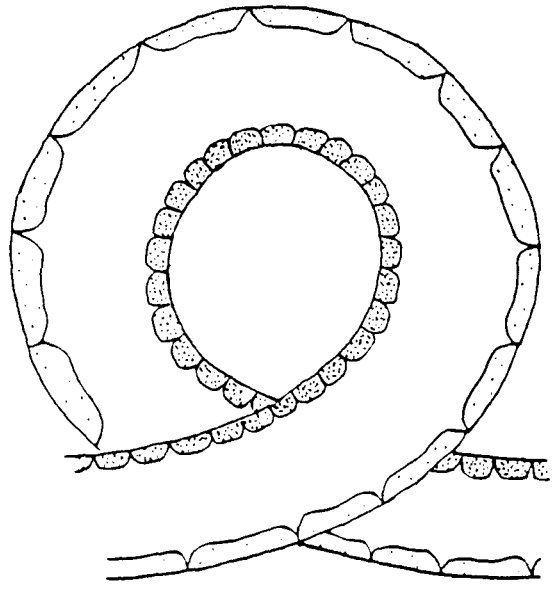


Fig. 7.22(b): Diagram depicting the state of contraction of muscles in a nematode which is coiled. The ventral muscles are in asymmetrical contraction along the length of the worm.

T. spiralis, which like the late third-stage larvae of N. dubius live coiled in cysts, also move using coiling-uncoiling movements (Despommier, 1982).

In comparison, larvae of Ancyclostoma tubaeforme migrate through their host, and no change was observed in their movement following infection (Croll et al., 1975). While it is possible that this nematode remains highly mobile after infection so that it can carry out its migration, this experiment remains open to an alternative interpretation. Croll et al. (1975) infected mice, rather than the natural host, with A. tubaeforme, and in mice these worms migrate to the lungs where they may remain infective for some months. Because the larvae studied by Croll et al. (1975) were still infective, one must assume that development had not resumed. Thus, the worms cannot be considered to have completed the transition to parasitism. If behavioural changes are to occur in infective-stage larvae, it seems likely that they will do so only after development has resumed in the new host. So the absence of a change in the behaviour of A. tubaeforme may represent an experimental artefact.

A comparison between the third-stage larvae of N. dubius and N. brasiliensis following infection would be of great interest. Late third-stage larvae of N. dubius lie coiled in cysts, while the larvae of N. brasiliensis typically migrate through the lungs of the host before taking up residency in the lumen of the intestine (Smyth, 1962). Evidence suggests that artificial infections of N. dubius can follow the same route of infection as N. brasiliensis (Chaicumpa et al., 1977) so it is possible that the larvae remain active until they locate a site suitable for growth.

Although it is possible, therefore, that the behaviour of infective larvae alters in response to an environmental stimulus, it

seems more likely, on the basis of this study, that the larvae of N. dubius undergo structural changes as a result of resuming development, and these structural changes in turn influence the behaviour of the worms. Thus worms might remain in the small intestine because they "recognize" it as a site suitable for growth (Sukhdeo & Croll, 1981), but they might move less efficiently following infection for other reasons, such as structural changes to the body wall.

In N. dubius, there were structural alterations in the body wall which could be related to the behavioural changes. Because the changes in movement occurred before the old cuticle separated from the epidermis, "slip" between the two structures could not be responsible for the inability of larvae make undulatory movements.

An outstanding change in third-stage larvae of N. dubius as they began to grow in the host, was observed in the amount of muscle fibre (Table 7.2, Figs 7.4 to 7.7). As the worms grew bigger in diameter, the body wall was weakened. The diminished somatic musculature is presumably capable of generating less force against the cuticle and internal hydrostatic skeleton. Indeed, the few late third-stage larvae to make undulatory movements appeared to have to work harder than infective-stage larvae, because the wave length of their tracks was shorter than those of infective-stage worms (see Appendix 4; Wallace, 1969).

The structure of the muscle fibres seen in Figs 7.8 and 7.18 is similar to micrographs of degenerating muscle present during moulting in Heterodera arenaria (Johnson et al., 1970), and therefore are not considered to be an artefact. Indeed Bird (1968) described muscular atrophy in the second-stage larvae of M. javanica after they had invaded a host.

The reasons for the breakdown of the somatic musculature of at

least some nematodes at the time of moulting is unclear. Davey (1965) proposed that the muscle tissue contributed precursors to the forming cuticle. Johnson et al. (1970), on the other hand, associated muscular atrophy with the quiescence, or lethargus, characteristic of moulting in nematodes but suggested no further role. But very little is known about lethargus; for example we do not know how widespread the phenomenon of muscular atrophy is, nor indeed whether it is a consequence, or the cause of the phenomenon.

Apart from the decrease in the amount of muscle fibre, the cuticle of third-stage larvae was thinner and more diffuse than that of infective larvae. Loss of the striated material from the basal zone, alone did not change the way in which larvae moved because worms in which the striated material was less pronounced could sometimes leave sinusoidal waves imprinted in agar. While these results are in no way conclusive, they cast doubt on the notion that the striated material is solely responsible for the tensile strength of the cuticle used in movement (Wright, 1968).

Factors other than the structure of the body wall would, of course, affect whether or not the parasitic stages were active on agar plates. Temperature, for example, was of primary importance. Where the temperature dropped below 26°C, the larvae coiled up without moving. At temperatures between 30 and 37°C, on the otherhand, the parasitic stages were active.

Thus when N. dubius infected a host, new gene-sets were presumably switched on which led to the resumption of development. The new gene-sets seem likely to initiate feeding and growth in these worms, but the behavioural changes seen in N. dubius are probably the result of structural alterations to the body wall, in particular, to the somatic musculature.

Very little is known about the infective-stage and the way in which development is resumed (Rogers & Petronijevic, 1982), but detailed studies of this sort offer valuable insights into the infective process. Much work has treated infective-stages as "black boxes" in an effort to determine the effect of various stimuli on development (Rogers & Sommerville, 1960; 1963; 1968). This study on the transition of free-living larvae to parasitism has highlighted some interesting details of the events set in motion following infection: the black box is slowly opening up.

CHAPTER 8

CONCLUSIONS AND GENERAL DISCUSSION

The parasitic nematode, N. dubius, emerges from this study as an organism capable of a diversity of form. It is polymorphic. Each time N. dubius moults, a new and different cuticle is formed, suggesting that alternative sets of genes are brought into operation.

The cuticle of N. dubius is an extracellular structure which forms, in part at least, by self-assembly of precursor molecules synthesized and secreted by the epidermis. And because the epicuticle is a laminate structure, it may be thought of as an envelope. Very little is known about the composition of many of the structures which were considered by Locke (1982) to be envelopes. Apart from being layered, extracellular structures, it is possible that they have little else in common. Nevertheless, the concept is an interesting one, and useful comparisons were made between the epicuticle of adult N. dubius and the outer membrane of Gram-negative bacteria in this study (see Chapters 5 and 6).

The structure of the cuticle of N. dubius appears to be related to the environment in which the worm lives and, in particular, to parasitism. Outstanding structural differences were observed between the cuticle of the parasitic stages (that is, the fourth-stage and adult cuticles), and that of the free-living forms, namely the first-, second- and third-stage cuticles. In the free-living worms the most obvious feature of the cuticle were two longitudinal ridges, or alae, which ran along the sides of the animal. On the other hand, the cuticle of the

parasitic stages contained a series of longitudinal ridges. In addition, a dense surface coat was only present on the parasitic worms.

Furthermore, when N. dubius was subject to a sudden change in environment during infection, the structure of the third-stage cuticle and the composition of its epicuticle altered. These changes emphasize that the cuticle is a dynamic structure. But the cuticle is extracellular, and must be considered to be non-living because it does not contain its own genetic material.

The capacity to alter extracellular structures is apparently widespread. The outer membrane of Gram-negative bacteria is extracellular, and its molecular composition can alter under the direction of the underlying cell and cell membrane (Bayer, 1979; Ghuysen, 1977; Hammond et al., 1984). In addition, the layer of wax on the surface of some leaves is also in a dynamic state. Although the wax provides an inert, protective surface for the leaf, extensive changes and exchanges take place between the wax and the epidermal cells beneath it (Cassagne & Lessire, 1975).

The mechanism by which the nematode cuticle alters between moults is unknown. Presumably the changes are under the control of the epidermis because the epidermis seems responsible for the synthesis and secretion of the precursors for the new cuticle. But how the epidermis directs the loss of components from the epicuticle, or arranges the insertion of new molecules into the epicuticle, is not known.

Samoiloff (1973) proposed that labelled proteins can move through the cuticle between moults, and suggested that the striated material might represent a channelling system. Alternatively, Anya (1966) identified RNA within the cuticle using methyl green-pyronin Y indicating that proteins might be translated outside the epidermis. There is no evidence that the type of enzyme generally involved with

membrane transport is associated with the epicuticle (Sayers et al., 1984), but the epicuticle of some nematodes contains intramembrane particles (see Chapter 5; Bird, 1984; Lee & Bonner, 1982) which could represent proteins that might be involved in membrane transport.

The infective third-stage larvae of N. dubius is particularly interesting because it makes the transition to parasitism. Not only does the third-stage cuticle alter when the infective larva becomes parasitic, but the epicuticle also exhibits some unusual properties prior to infection. The infective-stage larva is the only stage in which the epicuticle is multilaminate and does not exhibit a net negative charge.

In many studies of the infectious process in nematodes, the infective-stage has been thought of as a "black box". Much has been said about the stimulus that re-starts development and the initial changes that follow, such as exsheathing and hatching, but little is known about the other events which are associated with the resumption of development.

In this study some details of the developmental events occurring in N. dubius following infection were noted. Within 24 hours of infection, reserves of lipid and glycogen present in infective-stage larvae prior to infection, were judged to have diminished. And, unlike the infective-stage larvae, in worms that been in the host for approximately 48 hours a functional intestine could be detected. Moreover the worms increased in diameter as they developed into fourth-stage larvae, and the third-stage cuticle became thinner and more diffuse.

There were also alterations to the behaviour of the third-stage larvae associated with the transition to parasitism. These behavioural changes appeared to be caused largely by a decline in the somatic

musculature. Worms removed from the host 6 hours after infection or later, moved less efficiently over agar than did the free-living infective-stages.

However, such behavioural changes do not explain why larvae enter a "stationary phase" in the muscularis of the small intestine (Sukhdeo et al., 1981). Third-stage larvae still appear capable of migrating through the host until 24 hours after infection at least, because if infective-stages are injected intravenously into the tail of a host instead of being given orally, they will reach the small intestine via the lungs (Chaicumpa et al., 1977). Assuming that larvae from both routes of infection undergo similar developmental changes, which seems a reasonable assumption, then larvae must remain in the small intestine for reasons other than the changes in somatic musculature detected in this study. Presumably the third-stage larvae "recognize" the small intestine as a suitable site for further growth, and remain there (Sukhdeo & Croll, 1981).

This study of the composition of the epicuticle of N. dubius must be regarded as being a preliminary one. It served to highlight fundamental differences in the composition of the epicuticle of the different stages of N. dubius. Freeze-fracture studies, taken together with the results of staining and treatment with organic solvents, indicate that an important part of the composition of the adult cuticle of N. dubius, at least, might be a bilayer of lipid in some form, possibly lipopolysaccharides. Protein and/or glycoprotein also seems to play a major role in the composition of the epicuticle.

The third-stage epicuticle differed from that of adult worms. The third-stage epicuticle showed a marked preference to cross-fracture when freeze-fractured so that the interior of the epicuticle was not revealed by this method. Moreover, the surface of third-stage larvae did not

stain with ruthenium red, alcian blue or cuprolinic blue, while that of the adult worms was enhanced with these dyes.

As well as highlighting variations in the composition of the epicuticle within a species, differences were also noted between species. While the epicuticle of adult N. dubius was disrupted by a strong reducing agent, for example, that of some stages of C. elegans was not (Cox et al., 1981a). But many questions relating to the composition of the epicuticle remain unanswered because the techniques were not adequate.

The methods used in this study were relatively crude but had the advantage of being able to visualize the effect that various chemicals had on the surface of N. dubius. This type of study (see also Murrell & Graham, 1982; Murrell et al., 1983) emphasize that our understanding of the epicuticle of nematodes is still at the Naturalist's "look-see" stage. A different approach has involved analyzing the surface proteins that label with radioactive iodine (Parkhouse et al., 1981), but this too has limitations. In some instances the label can penetrate the cuticle, and there are difficulties in visualizing the label within the epicuticle because autoradiography does not offer sufficient resolution (Forsyth et al., 1981b; Marshall & Howells, 1986; Philipp et al., 1984). Furthermore, not all surface antigens are detected by the method and specificity of alternative methods of iodination varies between different stages of a species (Bashong & Rudin, 1982; Maizels et al., 1983; Marshall & Howells, 1985; Parkhouse et al., 1981; Sutanto et al., 1985).

An important and difficult question that must be faced is how to relate the composition of the nematode epicuticle to the survival of these animals. A great deal of interesting work has focused on those proteins present at the surface of parasitic nematodes which evoke an

immune response. And although Phillip and Rumjaneck (1984) noted that there might be relationship between the changing antigenic proteins on the surface of T. spiralis and N. brasiliensis, and the transition to a new environment, more often than not the emphasis of this work is on the reaction of the host to the nematode, rather than on the reaction of the parasite to its host. Some of the obseravtions in this thesis suggest there is a might be much to be gained from the latter approach.

Appendix 1

An attempt was made to analyze the distribution of negative charge on adult N. dubius using microprobe analysis.

It was first necessary to verify that X-ray emissions, collected with an energy dispersive system from two sputter-coats, are be proportional to each other, whatever the topological variations on the surface of the specimen (ap Gywnn, 1981). The surface of a block of resin was made uneven by scratching, and then sputter-coated with iron and with copper. Two thicknesses of copper were used.

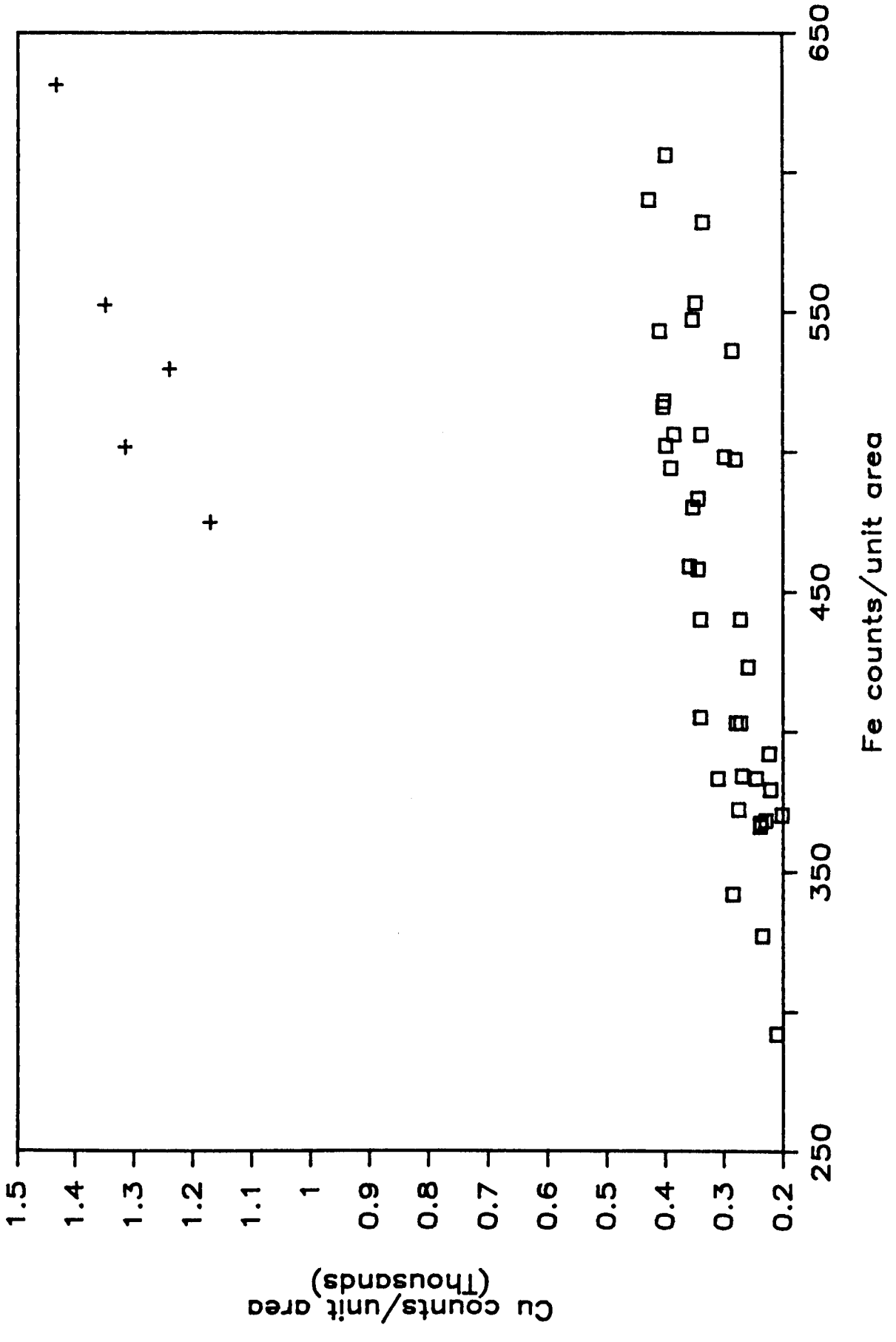
In keeping with the results of ap Gywnn (1981), the X-ray emission from the 2 metals was proportional over an irregular surface (see Graph A).

To examine the distribution of negative charge on adult male N. dubius, worms that been labelled with cationized ferritin were sputter-coated with copper (see Section 6.2.1). Results of microprobe analysis of four worms that had been labelled with cationized ferritin, and two unlabelled control worms are shown in Graph B. It is clear that there is no trend in the number of molecules of cationized ferritin adhering to the worms, except that a negligible amount bound to the bursa.

(Appendix 1 continues...see over)

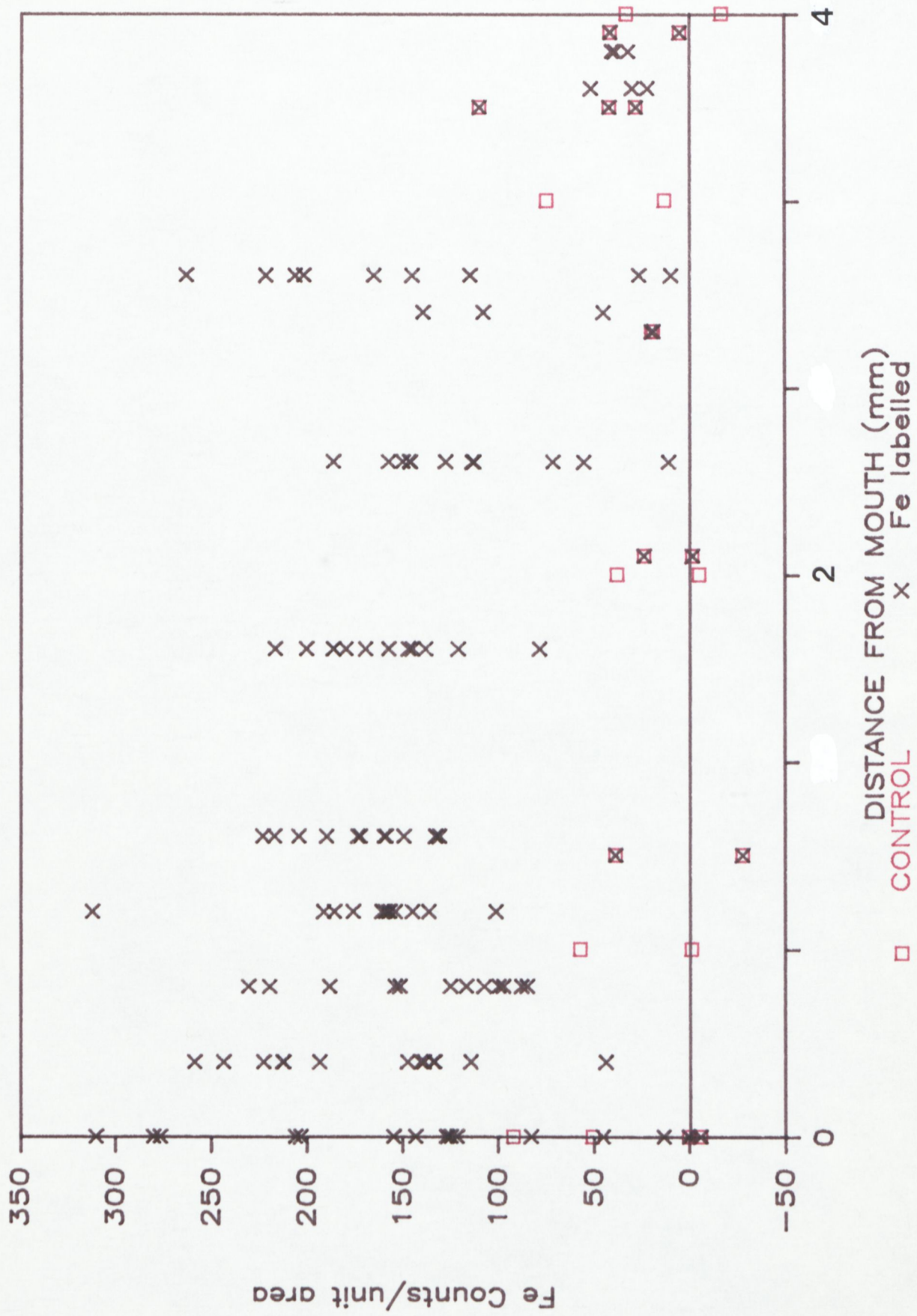
(...Appendix 1 continued)

Graph A: Iron (Fe) counts made against copper (Cu) counts on an uneven surface sputter-coated with the two metals. The thicker Cu coating is denoted by +.


Cu counts/unit area
(Thousands)
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
250
350
450
550
650
Fe counts/unit area

(...Appendix 1 continued)

Graph B: Iron (Fe) counts made along the length of 4 labelled male Nematospiroides dubius and 2 unlabelled worms. Data were pooled. Three counts were made over 0.045mm^2 at each point examined. Note that the counts of Fe on unlabelled worms ranged from -27 to 110 per unit area.


Fe Counts/unit area
350
300
250
200
150
100
50
0
-50
0
2
4
DISTANCE FROM MOUTH (mm)
× Fe labelled
□ CONTROL

Appendix 2

Statistical comparison between the length of infective larvae (n = 50) and the length of third-stage larvae (n = 50) removed from the host 64 hours after infection. Data exhibited homogeneity of variance using Bartlett's test and were analyzed using a t-test.

$$t_{observed} = 7.6$$

Critical $t_{.02\ (98)} = 2.617$, which is less than the observed value. The mean lengths of the two groups of larvae, therefore, are significantly different ($P < .02$).

Appendix 3

Statistical comparison between the distance moved by infective larvae of Nematospiroides dubius and third-stage larvae that were removed from a host 6 hours post infection. Data were analyzed using a Mann-Whitney U-test.

Distance moved (mm) by :

Infective larvae: 18, 27, 32, 45, 51, 53, 60, 69, 72, 72.

6 hr post infect: 2, 10, 10, 17, 20, 21, 21, 25, 40, 53.

$U_1 = 87.5$, $U_2 = 12.5$.

Critical $U_{.002\ (10,10)} = 90$ and $U_{.01\ (10,10)} = 84$.

Therefore the distance covered by infective larvae was significantly different to that covered by larvae removed from a host 6 hours after infection ($.01 > P > .002$).

Appendix 4

Statistical analysis of the relationship between the time that third-stage larvae were in the host, and the wavelength (λ) and amplitude (A) of the wave pattern they produced on agar.

The mean λ ($\pm$ standard error [SE]) and A ($\pm$ SE) of wave-like tracks left by infective larvae of Nematospiroides dubius and third-stage larvae removed from the host 6, 18, 48 and 72 hours post-infection (pi).

Stage of development	Wavelength (mm) $\pm$SE	Amplitude (mm) $\pm$SE	No. of sets of tracks measured
Infective	0.21 $\pm$ 0.004	0.11 $\pm$ 0.003	4
6 hrs pi	0.18 $\pm$ 0.003	0.09 $\pm$ 0.02	4
18 hrs pi	0.17 $\pm$ 0.03	0.08 $\pm$0.003	2
48 hrs pi	0.2 $\pm$ 0.004	0.08 $\pm$0.003	2
72 hrs pi	0.18 $\pm$ 0.004	0.08 $\pm$ 0.003	1

Data were analyzed using a two-way anovar. The data exhibited homogeneity of variance after logarithmic transformations using Bartlett's test.

(Appendix 4 continues...see over)

(...Appendix 4 continued)

TWO-WAY ANOVA TABLE

Source of variation	Mean square	Degrees of freedom	Variance ratio
Between λ & A for all treatments	70847.3	1	1584.08
Between treatments	886.052	4	19.8113
Interaction bet. λ & A	230.177	4	5.14655
Error	44.7245		

Critical $F_{(4,400)}$ = 3.3 at 1% level of probability.

The differences in wave form between different treatments was significant ($P < .01$). In addition, there was a significant interaction ($P < .01$) between wavelength and amplitude ($F_{4,400}$ = 3.36 at 1% level of probability).

There was a significant difference ($P < .01$) between wavelength and amplitude and all treatments ($F_{1,400}$ = 6.7 at the 1% level of probability).

Appendix 5

Lengths (mm) of third-stage larvae of Nematospiroides dubius removed from the host 54 hours after infection. (Some larvae were shorter than infective-stages, probably because these larvae were fixed and embedded before being measured. But because the worms in this study received the same treatment, comparisons were made between them).

Worms that left waves	Worms that left lots of wriggles	Worms that left a few wriggles	Worms that coiled up
0.3	0.55	0.5	0.5
0.37	0.37	0.57	0.57
0.42	0.5	0.3	0.43
0.38	0.6	0.53	0.65
	0.47	0.67	0.37
			0.5
Mean 0.37	0.50	0.51	0.49
S.E. 0.025	0.04	0.06	0.04

Data were analyzed using a one-way anova with unequal sample sizes. The data exhibited homogeneity of variance using Bartlett's test.

(Appendix 5 continues...see over)

(...Appendix 5 continued)

ONE-WAY ANOVA TABLE

Source of variation	Mean square	df	F
Between groups	0.019495556	3	2.0046
Within groups	0.009725521	16	

Critical $F_{(3\ 16)}$ = 3.24 at 5% level of probability.

The length of the worms was not a significant contributing factor to the way in which they moved but the sample size was too small for effective statistical analysis.

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