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An Ultrastructural Study of the Distal Epidermis and the Occurrence of Subcuticular Bacteria in the Gutless Tubificid *Phallodrilus albidus* (Oligochaeta : Annelida)

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Abstract

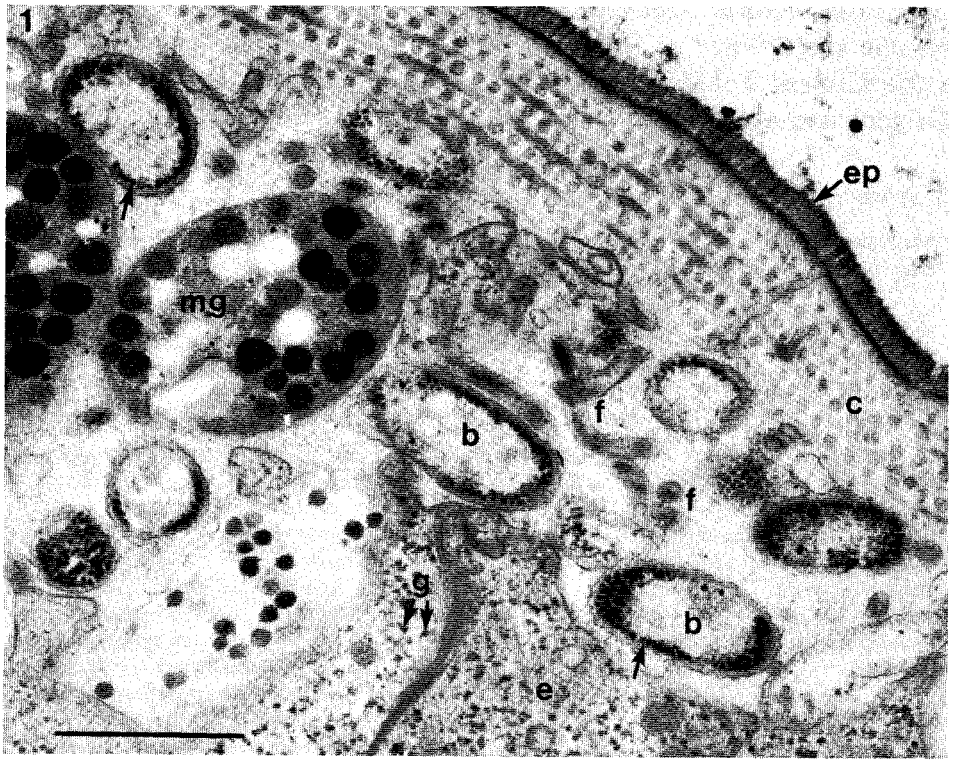
The typically annelid cuticle of *P. albidus* has an increased population density of epicuticular projections, but this is not thought to be related to its gutless condition. The irregular outline of the apical membrane of the epidermal cells is an inherent feature, possibly enhancing an absorptive function. The cuticle–epidermal interface is characterized by multigranular bodies, rod-shaped bacteria and filiform structures. The multigranular bodies are present in the postclitellar region only, thus coinciding with the chalkiness of living specimens. The bacteria occur throughout the entire length of the worm, their presence exaggerating the irregularities of the apical membrane. The bacterial cytoplasmic membrane, in contrast to the limiting membrane, was difficult to resolve. Between the two membranes is an amorphous zone. Storage granules of a carbohydrate-rich complex are located peripherally and the bacterial core contains a reticulum of DNA threads. Filiform structures, ultrastructurally similar to the bacterial peripheral region, are numerous, especially where the bacteria are carbohydrate-rich. Connections with the bacterial bodies were observed, but infrequently. The non-pathologic association between the subcuticular bacteria and the gutless tubificid is discussed.

Introduction

The gutless marine tubificid *Phallodrilus albidus* was described from littoral and sublittoral sands at Heron Island, on the Great Barrier Reef of Australia (Jamieson 1977; Erséus 1979*a*, 1979*b*). Ten further gutless species were subsequently recognized, notably from Bermuda (Giere 1979) and Florida (Erséus 1979*a*), bringing the total of anenteric and enteric species in the genus to 29 (Erséus 1979*a*). Two additional gutless species are known from other genera: *Inanidrilus bulbosus* Erséus (1979*b*) and *Coralliodrilus avisceralis* Erséus (1981), of which the latter is from Heron I.

Only a small minority of the 'gutless' *Phallodrilus* species show any vestiges of the original alimentary canal (Erséus 1981). In *P. albidus* the canal is totally lacking. In their gutless condition, these oligochaetes are similar to the Pogonophora. Although pogonophores have been the subject of numerous ultrastructural and experimental studies (see George 1977; Stewart 1979), no comparable investigations have been published on anenteric tubificid oligochaetes.

The absence of a gut necessitates dependence upon other systems for the absorption of nutrients. Transintegumentary uptake of low-molecular-weight organic materials has been demonstrated by A. J. and E. C. Southward in the pogonophores (reviewed by Stewart 1979). The ultrastructure of the epidermis of the Pogonophora (George 1977), which is very similar to that of the annelids, does not, however, reveal any features that could be considered as specifically enhancing the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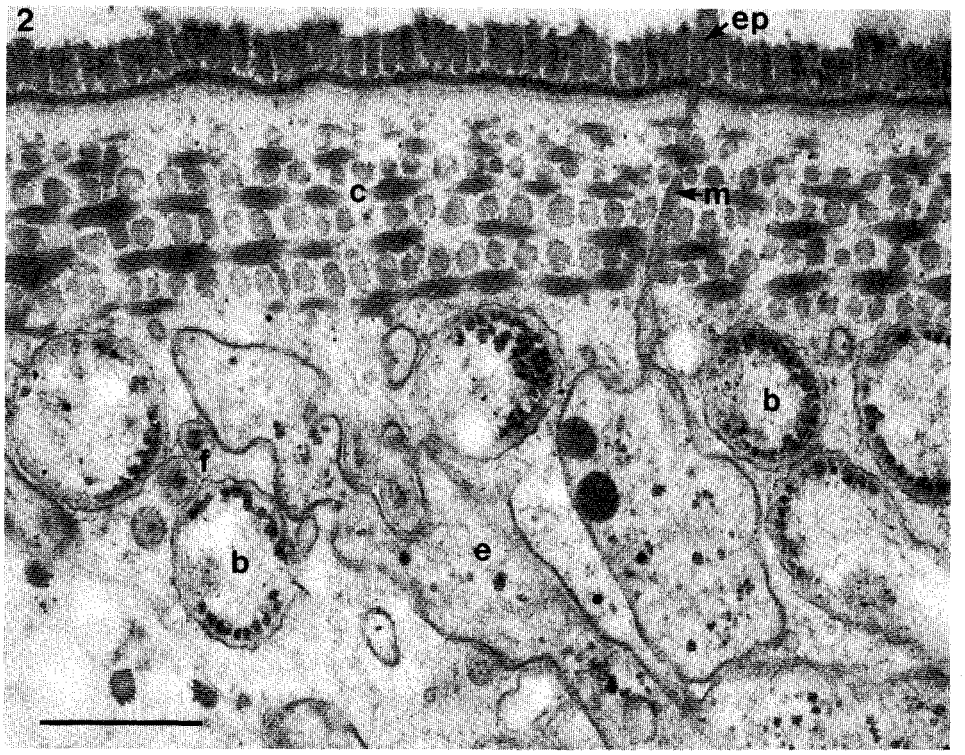
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functioning of the pogonophoran body surface as an absorptive one. This contrasts with the highly specialized microanatomical modifications present on the absorptive surface of gutless endoparasites; cestodes possess microtriches and acanthocephalans have pore channels (Chappell 1980).

A current study on the ultrastructure of *P. albidus* has revealed a number of features which differ from the typical tubificid condition, and the present communication reports those pertaining to the distal epidermal–cuticle region of the body wall.

Materials and Methods

Mature specimens of *P. albidus* were collected over a period of 5 years (1977–81) from coral sand under small coral heads on Heron Reef, Great Barrier Reef, Pacific Ocean. The animals were cut into anterior and posterior portions during fixation in 3% glutaraldehyde in 0·1M phosphate buffer (pH 7·2) at 4°C for 2 h. Following a phosphate buffer wash (as above) the tissue was post-fixed in 1% osmium tetroxide similarly buffered for 80 min, dehydrated in an ethanol series and embedded in Spurr's resin. Some specimens were processed omitting the osmium fixation stage. Routine 0·5–1·0-μm sections were stained with toluidine blue in 1% borax; ultrathin sections were stained with aqueous uranyl acetate followed by lead citrate prior to being viewed with a Philips 200 electron microscope at 60 kV.

For cytochemical localization of carbohydrate, the thiosemicarbazide–silver proteinate (TSC) method (Lewis and Knight 1977) was performed on ultrasections of glutaraldehyde–osmium fixed material. Localization of DNA was demonstrated by the Feulgen–silver methenamine technique (Lewis and Knight 1977) on ultrasections of tissue fixed in glutaraldehyde alone. In both techniques, sections of the median–posterior region of the body mounted on gold grids were used and appropriate controls were carried out.

Observations

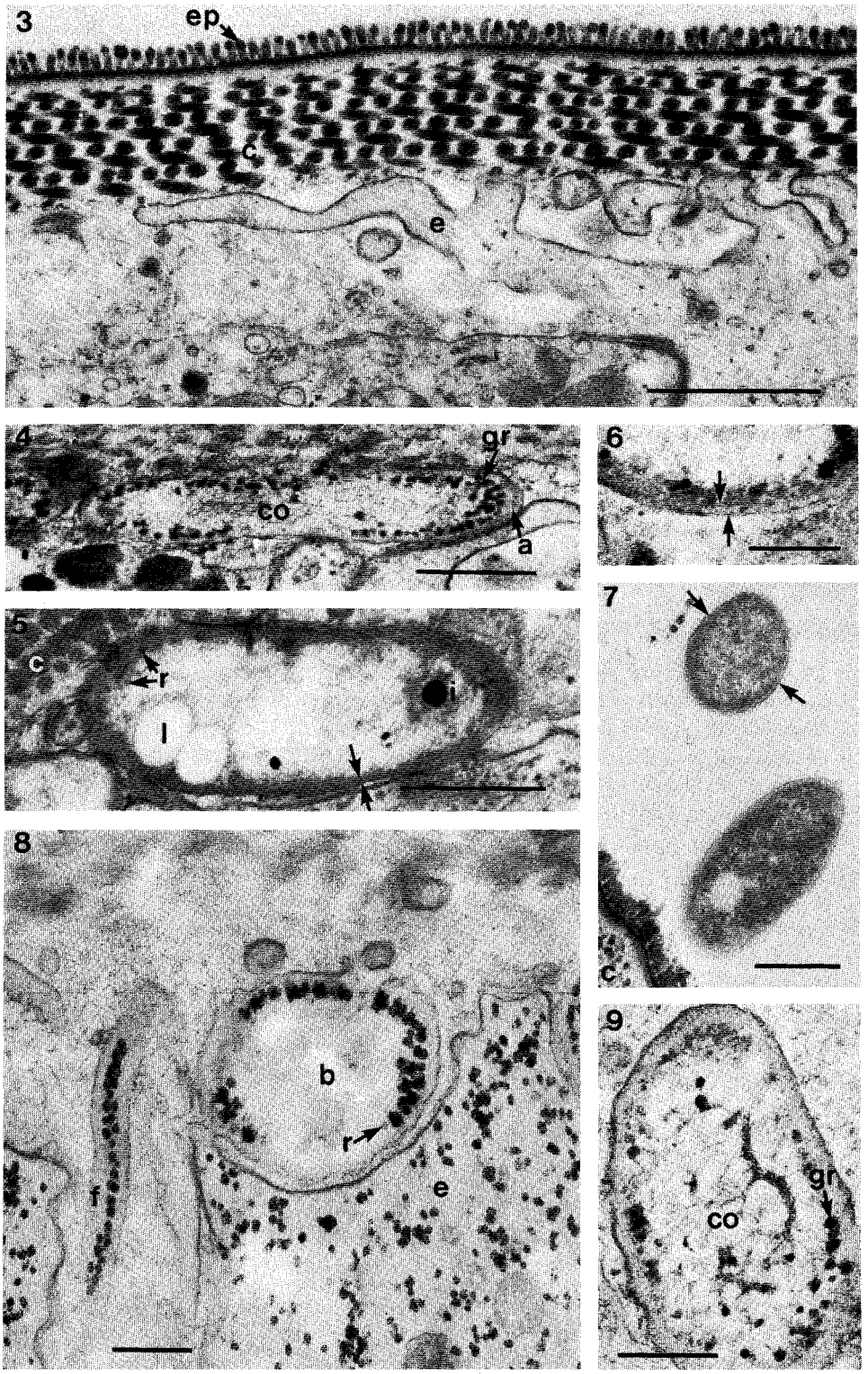
All the specimens of *P. albidus* used in this study were actively mobile at the time of fixation and displayed the white chalkiness in the region posterior to the clitellum, as described by Jamieson (1977).

The surface of the animal is covered by the cuticle (Figs 1, 2) 0·8–0·9 μm deep, with orthogonally arranged collagen fibres within the cuticle matrix. External to this lie the closely packed, membrane-bound, epicuticular projections (Figs 1, 2) which are 140–150 nm long, 40–48 nm wide and have a centre-to-centre distance of approximately 60 nm. These originate from the ascending microvillar extensions (Fig. 2) of the epidermal cells. In all oligochaetes previously studied the basal region of the cuticle matrix is in close apposition to the apical membrane of the epidermal cells, which, apart from the ascending microvilli, lies parallel to the outer surface of the body. Such an arrangement is not the case in *P. albidus*. The cuticle–epidermal interface is interrupted by the presence of extracellular structures of more than one type (Fig. 1). Consequently, the apical region of the epidermal cells is thrown into irregular folds (Fig. 2) which also occur in regions of the body where these structures are not particularly numerous (Fig. 3). On the basis of size, shape, ultrastructure and distribution, a clear distinction can be made between the types of subcuticular structure.

Fig. 1. The interface between the cuticle (*c*) and epidermis (*e*) is occupied by rod bacteria (*b*), filiform structures (*f*) and multigranular bodies (*mg*). Peripherally placed granules (arrows) within the bacteria resemble the glycogen granules (*g*) of the epidermis. *ep*, epicuticular projections.

Fig. 2. The subcuticular region, illustrating bacteria (*b*) and filiform structures (*f*) in transverse section. A fold in the distal epidermal epithelium (*e*) is shown. An epicuticular projection (*ep*) is seen originating from an epidermal microvillus (*m*) penetrating the cuticle (*c*).

Scale lines: Fig. 1, 1 μm; Fig. 2, 0·5 μm.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Bacteria

Rod-shaped bacteria (Figs 1, 2, 4), with many Gram-negative ultrastructural features, occur in the subcuticular region throughout the entire length of the animal. Occasionally they were observed intruding between the distal regions of adjacent epidermal cells. They are also present in the subcuticular position in those parts of the body where epidermal invaginations occur, for example, the ectal portion of the male duct. The bacteria are approximately 1·4 μm long and 0·4 μm in diameter.

Not all the components of the limiting region of the bacterium could be resolved with equal clarity. The outer trilaminar envelope is 7 nm in thickness but the cytoplasmic membrane of the bacterial body (also 7 nm thick) had considerably less affinity for lead staining and was less frequently seen in good section plane (Figs 5, 6). Between the two membranes is a zone of amorphous material approximately 15 nm deep. In the profiles where the cytoplasmic membrane could not be resolved this region appeared to extend for a greater distance internal to the outer membrane. The depth of the region comprising the two membranes and amorphous material (*c.* 30 nm) equates well with that of free-living bacteria occasionally seen external to the cuticle (Fig. 7).

In close association with the cytoplasmic membrane (and the amorphous zone when the membrane lacked clarity) lie electron-dense granules 30–32 nm in diameter (Figs 1, 2, 4, 6). These resemble the carbohydrate (glycogen) reserves of the epidermal cells (Fig. 1), and the reserves at both sites are TSC-positive (Fig. 8). The granules within the bacteria, but not those of the epidermal cells, gave positive reactions with the DNA test, and although negative in the control (non-hydrolysed) material this result is regarded as spurious (see the Discussion). In those bacteria located towards the anterior end of the animal the carbohydrate reserves are less numerous (Fig. 9). or absent (Fig. 5).

Granules of medium electron density and approximately 20 nm in diameter occur throughout the cytoplasm including the peripheral zone (Fig. 5). They are TSC-negative (Fig. 8) and are interpreted as the ribosomal components of the micro-organism. The central region contains a reticulum of threads (Figs 9, 12) which is DNA-positive (Figs 10, 11), as were the nuclei of the epidermal and other cells of the worm. At all these sites the non-hydrolysed control material was negative. Lucent areas within the core region of the bacterium were frequently present (Figs 1, 2, 5)

Fig. 3. Region of the distal epidermis showing a prominent epidermal fold (*e*). No bacteria are present (see text). *c*, cuticle; *ep*, epicuticular projections.

Fig. 4. Bacterium in longitudinal section containing peripheral granules (*gr*) and a core (*co*) of reticulate threads. Amorphous material (*a*) occurs external to the granules.

Fig. 5. Bacterium containing lucent areas (*l*), an electron-dense spherical inclusion (*i*) and medium dense granules interpreted as ribosomes (*r*), but lacking peripheral storage granules. The outer membrane and the cytoplasmic membrane are indicated by arrows. *c*, cuticle.

Fig. 6. Portion of a bacterium in which the trilaminar structure of the outer and cytoplasmic membranes (arrows) is evident. Amorphous material occurs between the membranes.

Fig. 7. Free-living bacteria external to the cuticle (*c*). Regions where the outer and cytoplasmic membranes are distinct are arrowed.

Fig. 8. TSC reaction. The peripheral granules of the bacterium (*b*), the core granules of the filiform structure (*f*) and the glycogen deposits of the epidermis (*e*) are positive. The TSC-negative granule (*r*) is interpreted as a ribosome. (Unstained section.)

Fig. 9. Bacterium in which the peripheral granules (*gr*) are less numerous. Note reticulate nature of the threads within the core (*co*).

Scale lines: Fig. 3, 1 μm; Figs 4, 5 and 7, 0·5 μm; Figs 6, 8 and 9, 0·2 μm.

and may represent another form of storage material. Highly electron-dense spherical inclusions (Figs 5, 12) also occur. The bacteria were not observed budding or in stages of spore formation.

Filiform Structures

Profiles of filiform structures in the subcuticular position were a common feature of the post-clitellar region of *P. albidus* (Fig. 1). Although present throughout the entire length of the worm, they were less numerous in the anterior region. These structures are of undetermined length, but profiles measuring at least 2 μm were observed. They are circular in cross-section, with a diameter of approximately 120 nm (Fig. 2). Their limiting membrane is 7 nm in thickness and internal to this is an amorphous zone similar to that observed in the bacteria. A cytoplasmic membrane internal to the amorphous zone was difficult to resolve.

The central core contains electron-dense granules 20 nm in diameter (Fig. 12) which, although smaller than the peripheral granules of the bacteria, gave similar cytochemical reaction, being TSC-positive (Fig. 8) and DNA-positive (Fig. 10). In conventionally stained sections there was some evidence of thread-like material in the core region (Fig. 12) but this was usually not visible due to the presence of the granules. No lucent areas or highly electron-dense spherical inclusions were observed.

Fig. 12 provides evidence of continuity of the filiform structures with the bacterial body, but such connections were seen infrequently.

Multigranular Bodies

These spherical, or subspherical, membrane-bound structures are 1·4–1·9 μm in diameter and contain numerous granules (0·20–0·28 μm diameter) of varying electron density (Fig. 1). The bodies are restricted to the post-clitellar region, their distribution coinciding with the observed chalkiness in the living animals. In addition to the subcuticular region, they also occur in intercellular positions deeper within the epidermis. The multigranular bodies are negative in the DNA test material (Fig. 10).

Discussion

The following discussion limits itself to those observations recorded for the cuticle and apical region of the epidermis of *P. albidus* which differ from the normal oligochaete pattern and which might be considered to relate to the anenteric condition.

The ultrastructure of the cuticle of *P. albidus* is similar to the basic organization in annelids (Richards 1978) and in pogonophores (George 1977). A minor difference recorded in *P. albidus* is the increased density of the population of membrane-bound epicuticular projections when compared with other microdriles, including tubificids (personal observations). However, this feature is not considered to be related to the nutrition of *P. albidus*. Richards (1980) was unable to demonstrate membrane-associated phosphatase activity (which normally correlates closely with known sites of nutrient uptake) at the level of the epicuticular projections in lumbricid oligochaetes which have been shown to transport dissolved organic nutrients across the integument (Richards and Arme 1979, 1980*a*, 1980*b*). The membrane of the ascending microvilli, which give rise to the epicuticular projections, was shown by Richards (1980) to possess such phosphatases, implying a change in membrane

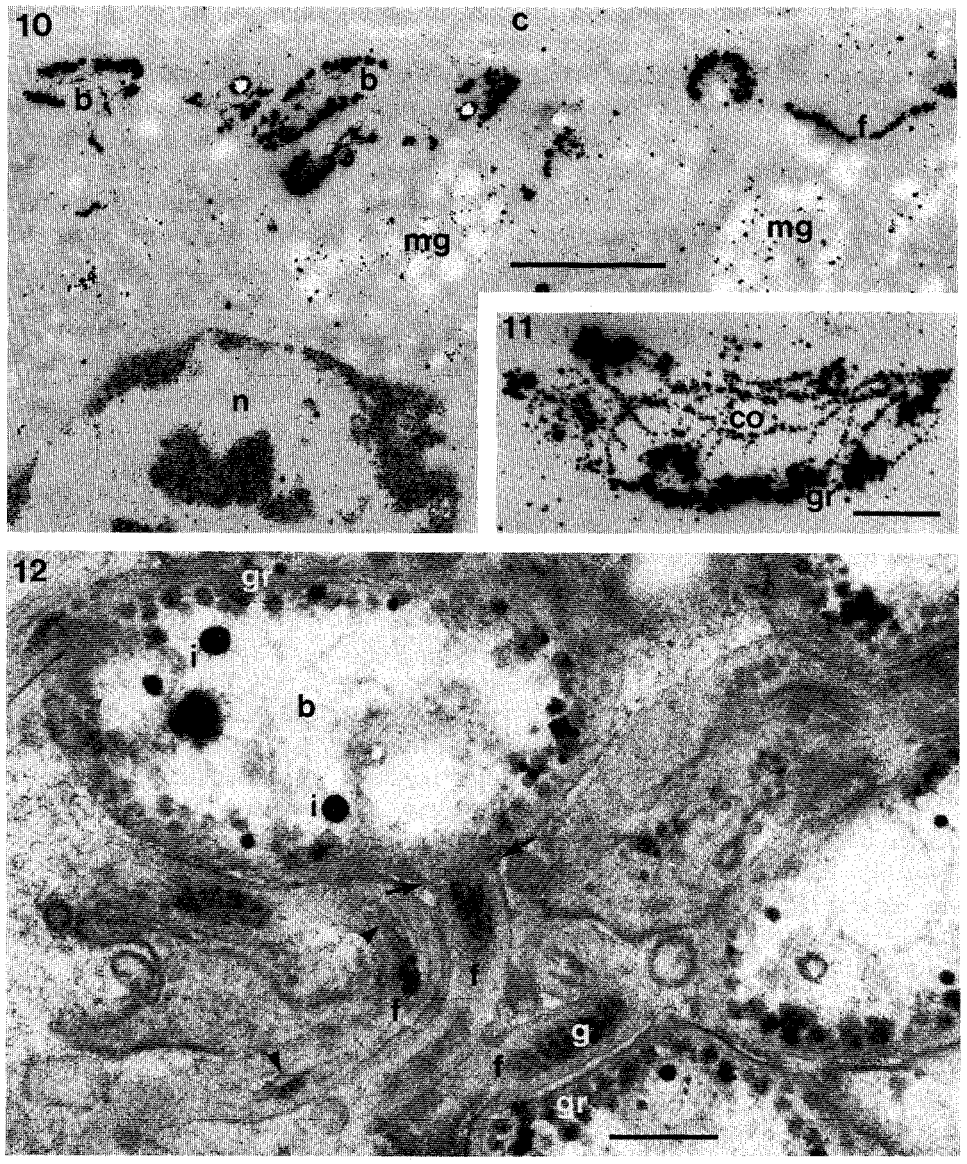

Fig. 10. DNA reaction. A positive reaction is seen in the bacteria (*b*), a filiform structure (*f*) and an epidermal nucleus (*n*), while multigranular bodies (*mg*) are negative. *c*, cuticle. (Unstained section.)
Fig. 11. DNA reaction. Positive bacterial sites include the threads of the core (*co*) and the peripheral granules (*gr*). (Unstained section.)
Fig. 12. Profiles of filiform structures (*f*) are shown, one of which is in continuity (arrows) with a bacterium (*b*). The core of the filiform extensions contains granules (*g*) which are smaller than those of the bacteria (*gr*) and evidence of thread-like material (arrowheaded). *i*, electron-dense bacterial inclusions. Scale lines: Fig. 10, 1 μm; Figs 11 and 12, 0·2 μm.

properties of the epicuticular projection once it is isolated from the 'donor' microvillus. This perhaps correlates with the acquisition of a protective function by the projections. The increased density of these structures in *P. albidus*, which lives in abrasive coral sand, is consistent with such an hypothesis.

The irregular outline of the apical membrane of the epidermal cells of *P. albidus* might be attributed to the presence of microorganisms, but it is also an apparently inherent feature of this species. Ahearn and Gomme (1975) demonstrated that the apical membrane of *Nereis diversicolor* is actively involved in the uptake of dissolved nutrients. The absorption of nutrients across the body surface is a common phenomenon in soft-bodied marine invertebrates (Stewart 1979) and it can be reasonably hypothesized that this pathway is functional in *P. albidus*. The small diameter of the body (approximately 0·2 mm) of the worm and the observed irregularities of the apical membrane, with the attendant increase in surface area, would favour such a surface-related route in an anenteric organism.

The presence of structures at the level of the cuticle–epidermal interface in *P. albidus* contrasts with any previous ultrastructural records of this site in oligochaetes (Richards 1977, 1978; Jamieson 1981) or the Pogonophora (George 1977).

The present study records that the subcuticular bacteria are a constant feature of *P. albidus* from Heron Reef collected over a 5-year period. The outer limiting trilaminar membrane of the bacteria was always clearly defined when the section plane was favourable, but good resolution of the cytoplasmic membrane of the bacterial body was rare. This difference would appear to be intrinsic to this particular bacterium, since in free-living bacteria (see Fig. 7) both membranes are clearly resolved. The existence of an outer membrane to the cell wall and the relative thinness of the amorphous (putative peptidoglycan) layer place the subcuticular bacterium in the prokaryote division Gracilicutes (see review by Costerton 1979).

The electron-dense reserves in the peripheral position within the bacteria are interpreted as storage granules of a carbohydrate-rich (TSC-positive) complex. The DNA-positive results at these sites are not regarded as representing a granular component of the bacterial DNA, or satellite DNA, but rather a false DNA reaction despite negation in control (non-hydrolysed) tissue. The specificity of the Feulgen-based DNA technique is not absolute (see Pearse 1968 for full discussion). The TSC and the DNA techniques both involve production of aldehyde from the substrate, but by different methods and with different degrees of ease. It is considered that the peripheral reserves in the bacteria of *P. albidus* have groups which react to both methods of aldehyde production; such substrates are not unknown (Pearse 1968). The present results, however, have shown that the bacterial peripheral carbohydrate reserve differs chemically from the glycogen granules of the tissues of *P. albidus*, since the storage products contrast in their reactivity to Feulgen hydrolysis.

The highly electron-dense spherical granules (Figs 5, 12) within the bacterial core are not inconsistent in appearance with polyphosphate (volutin, metachromatic) granules, and the lucent areas (Fig. 5) morphologically show similarities to poly-β-hydroxybutyrate granules (Shively 1974). Their composition is under investigation.

The filiform structures are interpreted as extensions of the surface of the bacterial cell, since connections were observed and there was no evidence that the relationship was pathogenic. Their size and ultrastructure preclude them from being any of the commonly occurring surface extensions of bacteria (e.g. flagella; pili; conjugation tubes; rod-like viruses as in certain mycoplasmas). The dimensions of the filiform structures relative to those of the bacterial body more closely resemble prosthecae, though these are often, but not invariably, in the polar position. The population density of the bacteria in the subcuticular position is moderately high, as is that of profiles of the filiform structures, particularly in the post-clitellar region. Connections were, nevertheless, rarely seen, and it is conjectured that not all the

bacteria possess filiform structures and that such extensions may be of considerable length. Their function is unknown, but the possibility that they are propagative cannot be ignored, since neither budding nor spore formation were observed. It was, however, noted that the filiform structures were most numerous in regions of the worm where the bacteria were rich in granular reserve material. They may, therefore, represent storage appendages.

The occurrence of multigranular bodies in subcuticular and intercellular positions throughout the epidermal depth in the post-clitellar region is reported. The failure to demonstrate DNA within them establishes that they are not microorganisms. This, therefore, precludes them from being candidates for a specialized, endosymbiotic nutritional role. Their distribution, however, coincided with the described chalkiness in the living worms (Jamieson 1977), and they will form the subject of a separate communication.

Since *P. albidus* is a gutless tubificid, the presence of subcuticular bacteria is of especial interest. There was no evidence of membrane-associated pigment in the bacteria and they are therefore presumed to be chemo-organotrophs. The bacteria resulted in no obvious pathology in *P. albidus*, and their presence in all specimens examined from a 5-year collection, together with their distribution throughout the length of the animal specifically in the subcuticular position, circumstantially militate against their being the result of an infection. Furthermore, their occurrence does not appear to be detrimental to *P. albidus* because all the tissues of the oligochaete showed unusually high levels of carbohydrate reserves.

The microorganisms possibly exist naturally as symbionts (i.e. in a heterospecific association irrespective of nutritional dependence). Some consideration must, however, be given to whether the coexistence might be commensal, with one obvious beneficiary, or whether it is mutualism with benefit to both individuals. The bacteria may simply be opportunistic, chemo-organotrophic commensals, living in the direct pathway of entry of the dissolved organic nutrients upon which *P. albidus* must depend. Situated in the subcuticular position, the microorganisms would equally benefit from any efflux of low-molecular-weight organic materials from the epidermal surface of the tubificid—a phenomenon known to occur in polychaetes (Ahearn and Gomme 1975; Stewart 1979).

The heterospecific association may, alternatively, be mutually beneficial, in that released metabolic products of the bacteria could be transferred to the epidermal cells of *P. albidus*, thereby supplementing the nutrition of the worm. Such an hypothesis has been advanced by Holland and Nealson (1978) for the role of the subcuticular bacteria in a range of echinoderms. The total nutritional budget of *P. albidus* must, however, be more surface-dependent than in echinoderms because of its anenteric condition. Furthermore, *P. albidus* does not possess nephridia. The excretory pathway must therefore also depend on the body surface. Neither the nature of the nitrogenous excretory products nor the osmoregulatory status of *P. albidus* are known. Nevertheless, whether the nitrogenous endpoint is ammonia or a less water-demanding product such as urea, the bacteria could possibly absorb and manipulate the excretory product (Lynch and Poole 1979) and liberate the 'recycled' nitrogen as amino acids which could be utilized by *P. albidus*. Such remobilization of nitrogen is known to occur in certain insect symbionts (Cornwell 1968). It is not without interest that echinoderms lack discrete excretory organs, and that Holland and Nealson (1978) recorded subcuticular bacteria in members of all the five classes of the Phylum.

The present ultrastructural study can do little more than document the presence of bacteria in the subcuticular position in *P. albidus*. Their role in the metabolism of this unusual oligochaete, lacking both a gut and excretory organs, must await physiological studies.

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