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AN ULTRASTRUCTURAL INVESTIGATION OF BODY WALL COMPONENTS IN *POROCEPHALUS CROTALI* (PENTASTOMIDA)

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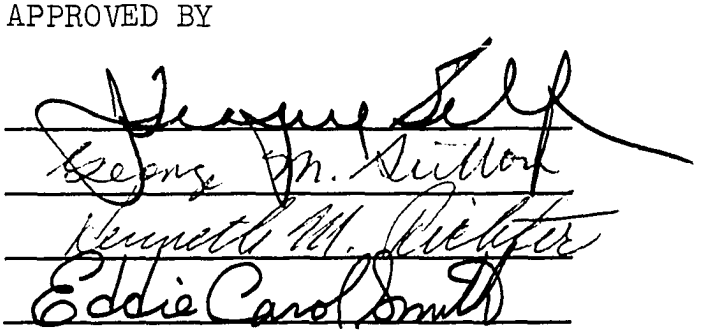
APPROVED BY



DISSERTATION COMMITTEE

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CHAPTER I

INTRODUCTION

The phylum Pentastomida is a little-known group of approximately 60 species of obligate parasites whose phylogenetic affinities are obscure. All life cycle stages are either endoparasitic or protected by an egg coat. *Porocephalus crotali* Humboldt, 1808 belongs to the Porocephalida, the more advanced of the two orders in the phylum.

P. crotali, like most other porocephalids, has an unusual life cycle in that the intermediate host, a mammal, is higher phylogenetically than the definitive host, a reptile. As shown by Esslinger (1962), infective eggs of *P. crotali*, coughed from the lung of the serpent host, are ingested by rodents with contaminated food and hatch into primary larvae. These penetrate the duodenal wall and undergo six molts while migrating through the viscera. The sixth stage, the infective nymph, is attained in approximately

ninety days in laboratory rats. When rodents are ingested by the definitive host, nymphs penetrate the intestine, migrate to, and mature in the lumen of the serpent's lung.

In spite of the great numbers of papers published on these worms, their biology is poorly understood. They are highly evolved helminths whose phylogenetic origin has long been enigmatic to those working on them. Like the Onycophora and Tardigrada, the Pentastomida possess characteristics of both the Annelida and Arthropoda (Nicoli, 1963; Osche, 1963; Doucet, 1965; Self, 1969).

The cuticles of pentastomes have been studied almost since the discovery of the group. Schulze (1932), using iodized zinc chloride after treatment with "diaphanol," found no difference between the cuticle of pentastomes and the chitinous exoskeleton of arthropods. Haffner (1924b) and Heymons (1935) described the cuticle as consisting of three layers, but Hett (1924) and Doucet (1965) could discern only two. The latter proposed that the third layer of Haffner and Heymons was an extremely thin one, composed of bacteria in host mucus, and occasionally observed attached to the cuticle following fixation "rapidly after capture." Doucet found that the cuticles of a number of species varied in thickness from five to fifty microns. The exocuticle-to-endocuticle thickness ratio varied from 1:4 to 1:9.

Doucet (1965) described the cuticle as being secreted by columnar cells rich in glycogen. He found the exterior

cuticle to be continuous with an interior cuticle in the foregut, hindgut, and genital openings. Doucet reported that in Armillifer armillatus both layers of cuticle in adults and nymphs are weakly positive to PAS (Lillie and Greco, 1947), PAS-ptyaline (Lison, 1960), PAS after three hours in ptyaline, and PAS-acetylation-KOH (Lillie, 1954). In the nymphs, weak positive tests were obtained in both cuticular layers with Bauer's Test (Bauer, 1933 modified per Lison and Vokaer, 1949) and Bauer's-ptyaline (45 min. at 37°C; Lillie, 1954).

Adults of Railietiella boulengeri are PAS and PAS-ptyaline negative in the exocuticle but weakly positive in the endocuticle, while Bauer's and Bauer's-ptyaline tests are negative in both layers (Doucet, 1965).

In light of the uncertainty of the efficacy of Bauer's and the PAS tests in the demonstration of chitin (Runham, 1961 and 1962; Salthouse, 1962), it seems safest to depend upon the method of Schulze (1932) for the demonstration of chitin in the pentastome cuticle. (This method is criticized however, by Richards, 1951.)

Doucet's results in the above tests might be due to polyhydroxyl or polycarboxyl mucosubstances occurring in complex with the chitin or, perhaps, the result of a large number of terminal N-acetyl-glucosamine residues in the chitin itself (Runham, 1962).

Pentastomes have no demonstrable excretory or respiratory systems and the cuticle is a logical place for these

activities to occur. Also, P. crotali nymphs are tissue dwellers while adults live in the lumina of lungs of the host. Little is known about changes in the cuticle and epidermal cells relative to this change from a tissue to an aerial environment.

In summary, although the cuticle of pentastomes has been studied, its true nature and the biological significance of its role in the host-parasite interrelationship are far from having been completely elucidated. The purpose of this study was the investigation of the body wall of P. crotali in the hope of learning more about its morphology, function, and mode of development. It marks the first application of the technique of electron microscopy to the body wall of a member of this phylum.

Components of the body wall of adult and infective nymph Porocephalus crotali are described. The significance of the findings, and their relationships to reports in the literature dealing with related organisms, is discussed. The mechanism of molting in adults is described. Possible modes-of-action for two sensory structures are presented.

CHAPTER II

MATERIALS AND METHODS

A. Biological Materials

P. crotali adults and eggs were obtained from naturally infected western diamondback rattlesnakes (Crotalus atrox, Baird and Girard) collected in Blaine and Comanche counties, Oklahoma. Nymphs were recovered from laboratory mice infected per os with eggs.

B. Transmission Electron Microscopy Procedures

Tissue was fixed three hours in 3% Glutaraldehyde and postfixed two hours in 1% Osmic Acid, both being buffered at pH 7.5 with Sorensen's phosphate and maintained at 4°C. Infiltration was performed directly from absolute ethanol through three increasing concentrations of epoxy to a pure epoxy-mix with catalyst. Vacuum assistance was used to enhance the final infiltration. A hard mixture (26g NSA to 4g DER 736) of Spurr's (1969) embedding medium was used. Three hundred to 500 A sections were cut on glass knives (43° nominal angle) and double stained for two minutes each in 1% uranyl acetate in 50% ethanol followed by a modified Reynolds

(1963) lead citrate. Thin sections were examined on an RCA-4A electron microscope operated at 100 KV.

CHAPTER III

OBSERVATIONS

A. General Features of Adult Body Wall

Cuticle

The cuticle of P. crotali is white to off-white and semitransparent at time of capture. The color changes to tan after specimens are stored in reptilian saline for six or more hours. Initially the cuticle is highly elastic, recovering fully after being stretched to twice its original size, and is very slightly hydrophobic. Both these attributes appear to decrease inversely with the time of storage prior to fixation. Neither acetone nor 50% ethanol resulted in any noticeable decrease in hydrophobia of the cuticular surface.

The cuticle is an acellular, non-living secretion of the underlying epidermal cells and is composed of three layers which differ in morphology. The outer epicuticle is 100 to 350 A thick. The intermediate exocuticle (two to eight microns thick) and the innermost endocuticle (eight to thirty microns thick) are separated from each other by an electron-dense layer some 600 A thick (Figs. 1 and 3).

Variability in the thickness of the exocuticle is due chiefly to outer surface irregularities. In areas free of irregularities its thickness varies from four to five microns.

The endocuticle is secreted by the subcuticular epidermal cells in successive lamellae (Figs. 1, 4, and 12). The interior (cellular) face of the endocuticle is very irregular, but its thickness variations depend primarily on the time since the last molt. From five to twenty-three lamellae were observed in this study, thickness varying from eight to thirty microns. Exocuticle-to-endocuticle thickness ratios thus varied from 1:2 to 1:7.

Subcuticular Cellular Layer

Three cell types were observed in the adult body wall: subcuticular epidermal cells, muscle cells, and apparent sensory cells.

Subcuticular epidermal cells are laterally attenuated (Fig. 4) and demonstrate irregular shapes that present a greatly enlarged surface area to the coelom (Fig. 2). The body wall is one cell thick except where cell boundaries interdigitate (Fig. 5). At these locations up to four or five greatly flattened cells may overlap between the coelom and the cuticle. Even where the body wall is one cell thick, extensive interdigitation may be seen (Fig. 4).

Muscle cells contain little undifferentiated sarcoplasm around the areas specialized for contraction or insertion into the cuticle. Mitochondria and endoplasmic

reticula (ER) are visible in these unspecialized areas, even as they are in the subcuticular epidermal cells (Fig. 5). Subcuticular muscle cells show extensive interdigitation with adjacent epidermal cells (Figs. 4 and 5). Insertion of fibrils occurs into the endocuticle (Fig. 5), and no apodemes or apophyses like those in arthropods are observed (Fig. 4).

Sensory cells will be discussed in conjunction with those found in nymphs in Section F below.

Coelom (Mixocoel)

The extensive coelom is of the mixocoel type (Osche, 1963). The irregular shape of the epidermal cells allows the coelom to extend inward close to the cuticle itself (Figs. 1 and 4). The coelom must function in the circulation of nutrient and waste materials and the shape of the cells enhances the area of surface contact between the cells and the coelomic fluid. No basement membrane in the classical sense is present between the epidermal cells and the coelom.

B. Ultrastructural Features of Adult Body Wall Components

Cuticle

The outer edge of the exocuticle appears to be indicated by an electron-dense line 100 to 200 A thick (Figs. 1, 7, 8, and 9) that appears to correspond to the epicuticular cuticulin layer of arthropods. The cuticulin is occasionally covered by a light homogenous layer about 150 A thick that may be of host origin, or a secreted wax layer.

This layer is seen in Figs. 7 and 9 but is absent in Fig. 8. These layers together comprise the epicuticle.

The exocuticle is differentiated into a superficial zone of increased electron density and a deeper zone of relatively less density (Fig. 7). Density in both areas is associated with fibrillar elements embedded in an electron-lucid matrix.

The relatively dense superficial exocuticular zone occasionally includes extra concentrations of electron-dense material in the form of cords (shown in cross section in Fig. 8) or more irregular shapes (Fig. 9).

Exocuticular fibrils measure 50 to 70 A in diameter by greater than 500 A in length. In the superficial zone, the fibrils occur in a tightly packed random array with a minimum of interfibrillar matrix. This arrangement blends gradually into the deep exocuticular zone which is characterized by increased amounts of matrical material and fibrillar organization into anastomatic bundles (Fig. 7).

Fibrils of the deep exocuticular zone are arranged in bundles which lie parallel to the surface of the cuticle (Fig. 7). Since both cross sectional and longitudinal views of these fibrils are seen, it is apparent that the bundles run at different angles (or perhaps curve) within a plane parallel to the cuticular surface.

The bundles appear to branch and intertwine, showing no lamellar organization. Sections occasionally form partial

or complete parabolic curves of fibrils within the cutting plane. This illustrates an internal organization of bundles similar to that encountered on a larger scale within endocuticular lamellae.

The intermediate cuticular zone is composed of tightly packed electron-dense fibrils. Little matrix is present, making resolution of the fibrils difficult (Fig. 3). Those seen measure 11 to 12 A in diameter and exceed 230 A in length.

The lamellar appearance of the endocuticle results from the arrangement of the chitin fibrils into ordered parabolic shapes (Fig. 12). The lamellae are variable in thickness, usually not exceeding one micron for any great distance, and are less pronounced or absent near the cells of the body wall (Figs. 1, 4, and 13). The fibrils are 30 to 40 A in diameter and exceed one micron in length (Figs. 12 and 13). As in the exocuticle, the fibrils are electron dense and are surrounded by varying amounts of less dense matrix. The dark areas in the lamellae occur where fibrillar material is closely packed (e.g. at the tails of the parabolas in Fig. 12). In some preparations the darkest zones occur in the opposite locations with respect to the parabolas (as pictured in Locke, 1964).

Fibrils are formed adjacent to the epidermal cells, but are usually not found in the parabolic shape (Figs. 1, 4, and 13). Some ordered orientation does occur at this level

however, as groups showing longitudinal and cross sections are visible (Fig. 13).

Subcuticular Cell Layer

a. General observations concerning subcuticular epidermal and subcuticular muscle cells.--Subcuticular cells rarely exceed two to three microns in thickness although subcuticular muscle cells generally extend further into the coelom and are often surrounded by epidermal cells making the body wall thicker at these points (Figs. 4, 5, and 18). Occasional canaliculi are seen extending from the coelom up into the epidermal cell layer (Fig. 5).

The cuticular surface of the epidermal and muscle cells are modified by microvilli (Figs. 5 and 6). The plasma membrane in this surface is 45 to 60 A thick. Microvilli are variably recumbent toward the main cell surface. They may be slightly bulbous, 0.03 to 0.07 micron in diameter, and do not appear to exceed 0.35 micron in length. No cell pores or other discontinuities in the plasma membrane are observable.

The cuticular surface of the epidermal cells becomes altered during the molting process as described in Section C below.

At the coelomic surface, the plasma membrane is the same thickness as that at the cuticular surface, but smooth and unmodified (Figs. 5, 10, and 11).

A 500 to 600 A layer of amorphic, homogeneous material of intermediate electron density coats the surfaces

of all cell membranes adjacent to the coelom (Figs. 2, 4, 5, 10, and 11). Unlike a basal lamina, this layer conforms to all surface irregularities of the cells, is found directly against the cell membrane, and is present on every type of cell exposed to the coelom. Terms such as basal lamina, glycocalyx, external lamina, etc. are all descriptively inaccurate here, and I shall refer to it as the coelomic boundary layer.

Epidermal cell interfaces are characterized by extensive interdigitation, smooth junctions, and two types of specialized junctions: desmosomes (maculae adherentes) and ladder-like junctions over large areas.

The smooth junctions are composed of standard 45 to 60 A membranes, closely apposed and parallel, separated by a 120 to 130 A layer of intercellular matrix (Figs. 5, 10, and 15).

Desmosomes occur regularly within 0.4 micron of the cuticular face of subcuticular intercellular junctions (Figs. 5, 6, and 15). They are absent at coelomic face junctions (Fig. 10). The junction is up to 0.2 micron long and is characterized by a swelling of the plasma membranes to 80 A and their separation by a 130 to 160 A electron-lucid matrix. The complex is traversed by extremely fine fibrils extending up to 400 A into the cytoplasm of each cell (Fig. 6).

The ladder-like junctions occur in sheets or fasciae, and are like the adherentes type of intermediate junction

(Fig. 5). Plasma membranes in these junctions are of the same thickness as those in smooth junctions and are separated by the same amount of electron-lucid matrix. In these junctions however, the matrix is crossed by fibrils (or membranes?) of the same thickness as the plasma membranes. The transverse fibrils occur at more or less regular intervals of not less than 160 A. These fasciae have not been seen to have larger lateral dimensions than 1.1 micron (Figs. 5 and 15).

All three types of intercellular junctions appear to occur with equal regularity at subcuticular epidermal-epidermal, epidermal-muscular, and muscular-muscular cell interfaces (Fig. 5).

b. Subcuticular epidermal cells.--Examination of thousands of thin sections revealed very few nuclei in the subcuticular epidermal cells (Fig. 11), although they are visible at the light level. This situation is suggestive of large, greatly attenuated cells, or perhaps even of syncitia.

Nuclei are irregular in shape, and up to 2.1 by 5.2 microns in size (Fig. 11). They appear to be uninucleolate, the ovoid nucleolus measuring some 0.63 by 0.87 microns. Faint chromatin aggregations are sometimes observed.

The nuclear membrane is distinctly tripartite, the inner 120 to 160 A membrane being separated from the 120 A outer membrane by a 200 A matrix. No nuclear pores are seen.

Mitochondria are common throughout subcuticular epidermal cytoplasm (Figs. 5, 11, and 15). Their shapes may

well be tubular, but sections grade from spherical, through oval to bar-bell shape. The largest dimensions observed were 1.4 microns by 0.4 micron. Apparent mitochondrial fusion is shown in Fig. 11, where the mass of mitochondrial material measured one by three microns.

Cristae are not tightly packed and consist of 60 to 80 A canals bounded by a 45 to 55 A membrane. I have observed no more than 15 cristae in a single mitochondrion. The external membrane is 30 to 40 A thick and is separated from the 45 to 55 A internal membrane by a 55 to 65 A matrix.

ER are also quite abundant in the epidermal cytoplasm and appear to be exclusively rough or granular in nature (Fig. 5). The ER may exceed 1.4 microns in length, and consist of a 40 A tubular membrane some 0.030 micron in diameter. Because of the attenuation of the cells, the ER are generally oriented parallel to the plasma membranes. Particles resembling ribosomes or beta-granules of glycogen (i.e. 120 A in diameter) are quite numerous and free in the cytoplasm, but follow no apparent pattern in their occurrence (Fig. 5).

Nothing resembling Golgi complexes have been observed by me in subcuticular epidermal cells. The cells often appear vacuolated (Figs. 4 and 13) and occasionally lipid bodies are seen in the cytoplasm (Fig. 15). No other cell inclusions or secretion products have been observed in the subcuticular epidermal cells.

c. Subcuticular muscle cells.--Muscle cells of this area are elongate and roughly perpendicular to the plane of the cuticular surface (Fig. 13). They occur at irregular intervals along the length of the worm, but never any closer than in Fig. 14 which illustrates the extreme anterior end of the worm.

The muscles are all striated in a normal skeletal muscle pattern (Fig. 16). The "A" band is 1.5 to 2.6 microns wide and the "Z" band is 0.4 to 0.7 micron wide. No "H" or "M" bands are visible, perhaps due to slight contraction prior to fixation. Sarcomere length varies from four to seven microns. The cells possess the "T-system" and agranular sarcoplasmic reticula typical of striated muscle cells (Fig. 19). Cross-sections through the "A" band demonstrate a normal arrangement of the actin and myosin filaments, the former being 73 A in diameter and the latter, 190 A (Fig. 17).

Distally, the subcuticular muscle cells differentiate into a specialized area for attachment to the cuticle (Figs. 5, 14, 15, and 18). This zone bears a superficial resemblance to vertebrate tendon. It is however, modified sarcoplasm that is intracellular and not separated from the myofilaments (Fig. 5) nor the sarcoplasm (Figs. 15 and 18) by a plasma membrane. I shall refer to the entire area as the attachment process of the muscle cell.

The dense 0.1 micron line that marks the end of the myofilaments is enigmatic. It is not thickened sarcolemma

and it may be a modified "Z" band (Fig. 14). This line is separated from the 280 A dense line at the end of the attachment process by at least a 190 A electron-lucid matrix. Occasional opposing discontinuities in both dense lines allow a few filaments to connect across the matrix (Fig. 21).

The attachment process extends from this point to the inner cuticular surface (Fig. 14). The length of the process has not been observed to exceed 16 microns.

It is not certain whether the attachment process is capable of contraction, but it does not appear that it is. Only one size fibril (180 A) is present and no striations occur. These are presumably tonofibrils separated from one another by matrical material some 80 to 85 A thick (Fig. 5).

At the cuticular surface, the tonofibrils appear to leave the attachment process through involuted cones in the sarcolemma (Figs. 15, 18, and 20). The tonofibrils extend up to 14 microns into the cuticle and intertwine with the chitin fibers of the endocuticle. As they extend further into the cuticle, the tonofibrils taper down to 120 A in diameter (Fig. 18).

The tonofibrils traverse the molting space prior to formation of a new cuticle (Fig. 22; see Section C below).

Most of the unmodified sarcoplasm in subcuticular muscle cells is crowded against the underside of the cuticle (Figs. 5 and 15). The plasma membranes of these muscle cells are identical in structure and attachment specializations to

the membranes of the subcuticular epidermal cells as described above. Mitochondria, lipid bodies and granular ER occur similarly as well. The mitochondria are apparently restricted to the subcuticular zone of the unmodified sarcoplasm and are not found in association with the contractile myofilaments (Figs. 5 and 15).

The absence of Golgi complexes and the presence of apparent free ribosomes or beta-particles also duplicates the cytoplasmic situation of the subcuticular epidermal cells (Fig. 5).

Nuclei in the subcuticular muscle cells occur in that portion of the sarcoplasm that is perpendicular to the plane of the cuticular surface. They are not however, in close association with the myofilaments.

The nuclear membrane is not at all spherical, since it is forced to follow the shape of the cell membrane to which it is closest; but it is further irregular beyond this association.

C. Ultrastructural Changes Characterizing the Molting Phenomenon in Adults

The molting process in adults is accompanied by manifest changes in the subcuticular cells. In the earliest stages of molting, modification of the cuticular surfaces of the cells occurs. The microvilli of the cell surfaces abutting the cuticle are elaborated into cell processes in increased numbers, sizes, and extending inward to greater

depths in the cells than the microvilli during intermolt. This process has not proceeded very far in Fig. 6.

The canals that result from cell process formation extend some three microns into the cytoplasm, show extensive branching, and occasionally enlarge into deep caverns within the cells (Figs. 23 through 26). The internal structure of the plasma membranes becomes highly modified as well. The outer membrane thickens to 150 to 200 A and is separated from the 55 A inner membrane by a 110 to 160 A matrix (Fig. 23). As molting progresses, a subcuticular space develops and widens between the cuticle and the epidermal cells. A progression of this process is also illustrated in Figs. 23 through 26.

The subcuticular space is filled with amorphous material of uncertain origin. If it is cuticular in origin, its removal from the cuticle does not appreciably change the appearance of that layer.

Tonofibrils of muscle insertions extend across the subcuticular space providing exoskeletal support, presumably until new cuticle is formed (Fig. 22).

The subcuticular cells become heavily laden with electron-dense material as molting progresses (Figs. 23-26), and it is possible that this is the origin of the material in the subcuticular space. There are, however, only bare hints of discontinuities in the plasma membranes through which this material would have to pass (Figs. 24 and 25).

The increased electron density of the epidermal cells is both dispersed and aggregated in form (Figs. 22-26). The concentrations (Figs. 24 and 25) are on the order of 100 to 125 A in diameter and could be glycogen beta-particles or perhaps free ribosomes. The nature of the more diffuse electron-dense material is not known.

The subcuticular space continues to enlarge prior to new cuticle formation. The space has been observed in excess of 2.2 microns. No specimens showing distinct formation of a new cuticle were obtained.

The increase in cytoplasmic density makes resolution of organelles in these cells very difficult. Inasmuch as these cells have to be very active metabolically, it is unlikely that the organelles disintegrate. Intercellular membranes and occasional mitochondria are discernible, and are like those organelles described above from non-molting subcuticular epidermal cells.

D. General Features of Infective Nymph Body Wall

Cuticle

Unlike adult cuticle, nymphal cuticle is in intimate contact with the tissue of its host (Fig. 27). The outer surface of the cuticle is shaped into spines which deform the plasma membranes of adjacent cells (Fig. 29).

Nymph cuticle is pale white to light yellow in color and semitransparent at time of capture. Little change in

color is observable after storage in mammalian saline for 36 hours or more. The cuticle is highly elastic, recovering fully after a 2X or 3X extension. Elasticity does not appreciably decrease with longer storage until death of the specimen, after which it becomes plastic.

The cuticle is at best very slightly hydrophobic. Neither 50% ethanol nor acetone resulted in any apparent decrease in the hydrophobia.

As in the adults, the cuticle of infective nymphs is a trilaminate acellular secretion of the underlying cells (Fig. 28).

The epicuticle is monolayered, some 400 A thick, and is elaborated into small spines.

The exocuticle is basically 0.5 micron thick, but varies from 0.3 to 1.4 microns with irregularities in its outer surface. Endocuticle thickness is more variable, depending on both the inner surface irregularities and the time since the last molt. Values in thickness from 5 to 9 microns were observed in this study. The two layers are separated from one another by a discontinuous, dense intermediate cuticular zone. Observed exocuticle/endocuticle ratios varied from 1:8 to 1:26.

Subcuticular Cell Layer

Two basic cell types have been observed in the infective nymph body wall: subcuticular epidermal cells and specialized cells that are thought to be sensory.

Subcuticular epidermal cells are columnar, typically 25 to 30 microns in height by no more than 11 microns in diameter. They show little interdigitation with adjacent cells. More-or-less spherical nuclei some six microns in diameter are located roughly in the center of the cells. Mitochondria, Golgi complexes, and ER are concentrated in the area adjacent to and distal from the nuclei (Fig. 27).

While the principal masses of the subcuticular epidermal cells are columnar, they show continuity with basal portions that appear to be pleomorphic (Fig. 27). The coelom is not evident here because of its rudimentary nature in nymphs. The nature of the relationships between the principal and basal masses of these cells is undetermined. Although the coelom was not observed, it can be said that, as in the adults, no basement membrane is present under the epidermal cell layer.

Subcuticular muscle cells were not observed in the nymph at the electron microscopical level although they are known to be present from light level investigations.

The so-called "stigmata" will be discussed in conjunction with those found in adults (Section F, below).

A bulb-shaped organ of infective nymphs is herein proposed to have a sensory function. The complex is composed of an outer cup-shaped cell and an inner, teardrop shaped, dense cellular element (Figs. 30 and 31). The complex is six to ten microns in diameter and nine to thirteen microns

in height. The inner dense element fills the cup-shaped cell which is from 0.5 to 1.7 microns thick. The dense element protrudes into the cuticle through a one micron orifice in the apex of the cup-shaped cell. The cellular element is characterized by a mass of dense material surrounded by a plasma membrane and containing many large vacuolar compartments. I observed no organelles in this material.

E. Ultrastructural Features of Infective Nymph Body Wall Components

Cuticle

The epicuticle in infective nymphs consists of a single 400 to 440 A thick electron-dense layer. It is elaborated into small secondary spines some 85 to 90 A high (Fig. 29).

The exocuticle of infective nymphs appears to be a nonfibrillar, homogeneous layer of intermediate electron density. Its outer surface is modified into distinct primary spinous processes some 0.4 micron high.

Exocuticular filaments appear to be extruded through occasional pores in the exocuticle and to extend outward to the host's cells (Fig. 28). The filaments are some 320 A in diameter.

An intermediate cuticular zone separates the exocuticle from the endocuticle (Fig. 29). This zone differs from its counterpart in the adult in that it is composed of widely separated cords 440 A in diameter oriented in different

directions within a plane parallel to the surface of the cuticle. Large areas of continuity exist between the exocuticle and endocuticle between the cords.

The endocuticle is composed of tightly packed 240 A fibrils in a relatively lucid matrix. The fibrils are oriented in the shape of waves, but remain nearly parallel to the plane of the cuticular surface. Lack of parabolic orientation of the fibrils precludes the appearance of lamellae (Fig. 28).

Subcuticular Cell Layer

The cell surfaces of subcuticular epidermal cells are elaborated into microvilli at the cuticular surface. The microvilli are more common at, if not restricted to, the lateral areas of the cell surfaces. The microvilli are recumbent, 0.17 micron in diameter, and exceed 0.5 micron in length. No pores or other discontinuities are visible in the cuticular surface membranes (Fig. 27).

Plasma membranes show little interdigitation with adjacent cells in any direction. Desmosomes occur in the lateral intercellular junctions near the cuticular surface. No other attachment specializations are seen, nor do desmosomes occur at any other location (Fig. 27).

Nuclei are ovoid, 3 microns by 4.5 microns in diameter, and are bounded by a 270 A inner membrane, up to a 400 A intermembranal matrix, and a 270 A outer membrane. Six hundred eighty A nuclear pores occur sparsely.

Karyoplasm is not differentiated into nucleoli and chromatin does not manifest itself in dense areas within the nucleus (Fig. 27).

Mitochondria are common, but rarely appear deeper in the cell than the level of the nucleus. Sizes of mitochondrial sections vary up to 3.8 microns by 0.8 micron. Cristae are 545 A in diameter, exceed 0.27 micron in length, and occur rather sparsely (Fig. 27).

Granular ER are generally 545 A in diameter and are common in the cytoplasm near the nucleus. They also occur in the distal cytoplasm among the mitochondria, but less frequently there. Occasional Golgi complexes are seen in the area of the nucleus. These consist of vesicles 0.10 micron in diameter and up to 1.4 microns long. The vesicles are neither straight nor arranged parallel to each other (Fig. 27).

The cup-shaped cell of the bulb-shaped complex is nearly devoid of organelles; a few mitochondria and aggregates of small vesicles are all that I have observed (Fig. 31).

Desmosomes and fasciae adherentes occur between this cell and the adjacent subcuticular cells, but not with the internal dense cellular element. The membrane of the outer cell is 110 A thick and is separated from the convoluted plasma membrane of the dense element by an amorphous layer of intermediate density between 275 A and 0.17 micron thick. This amorphous layer is contiguous with the exocuticle.

The plasma membrane of the dense element is tripartite, composed of two 55 A membranes clearly separated by a 110 A matrix. The internum of the dense cell is filled with amorphous dense material, homogeneous except for large vacuolar cavities that are electron-lucid.

F. Ultrastructural Features and Relationships of Cuticular "Stigmata" in Nymphs and Adults

General Observations

The cell complexes which have long been referred to as "stigmata" or cuticular glands contain two main cells in both infective nymphs and adults. In both cases also, these cells are distinguished by extensive microvillar-like specializations of their distal cell surfaces.

Both are observed at the external cuticular surface as a circular spot, hence the name "stigmata." Although this spot has long been referred to as a pore, it is shown herein to be filled with undifferentiated cuticular material, thus affording no effective opening through the cuticle.

"Stigmata" of Infective Nymphs

The continuity of the cuticle is interrupted by a circular spot 10 to 15 microns in diameter. The surface spot is composed of a central disc and a circumferential collar or hinge which changes from 0.3 to 1.7 microns in width from top to bottom (Fig. 32). Both the collar and the central disc are 2.0 to 2.5 microns deep.

The intermediate cuticular zone aggregates at the collar and this electron-dense material appears to be incorporated into the collar, making the two homologous (Fig. 35). The very edge of the so-called stigmata is typified by a thinning of the endocuticle and a thickening of the intermediate cuticular zone (Fig. 33). The central disc's exterior surface is identical to that of the nymphs' epicuticle and exocuticle and these two are thought to be homologous as well.

Laterally, the central disc is separated from the collar by an electron-dense zone of variable thickness. The internal face of the central disc is elaborated into extensions which fill the space between the cell processes of the underlying modified epidermal cells. Both the central disc extensions and the cell processes exceed 12 microns in length. The cell processes are tubular, 0.1 micron in diameter and are limited by a 55 A plasma membrane. They extend to within 2.5 microns of the exterior surface of the worm and contain some fibrillar internal organization, precluding their being called microvilli (Fig. 34).

The cytoplasm of the modified epidermal cells contains mitochondria resembling those described in standard subcuticular epidermal cells above (Fig. 36). The cells are uninucleate and the nuclei do not appear to have nucleoli. Dense chromatin areas occur within the amorphous karyoplasm. The double membrane is invested with nuclear pores 0.29 micron

in diameter (Fig. 32). Neither Golgi complexes nor ER have been observed in these modified cells.

In addition to the two large modified cells, smaller accessory cells are seen in the region beneath the collar (Figs. 32 and 35).

"Stigmata" in Adults

The central disc in adults is some 4.9 microns wide by 6.6 microns deep. Its electron-dense collar is generally four microns high by 0.9 to 1.3 microns wide. Cell processes of the two large cells are up to 0.16 micron in diameter and 10 microns in length. The plasma membrane over the cell processes is some 90 to 110 A thick.

Internally, the cytoplasm of each of the modified cells is essentially the same as reported above in the nymphs. The main difference in adults is that the central disc extensions are greatly reduced, leaving an infradiscal "space" between the disc and the cell processes. The space is filled with electron-transparent material. Fig. 37, a slanted transverse section through the infradiscal material, illustrates the cell processes in the material of the infradiscal space. Flattened accessory cells lie around the two modified cells, which contain several vacuoles.

Coelomic extensions some 0.16 micron wide extend into the accessory cells and penetrate to within 0.9 micron of the infradiscal space (Fig. 38).

LIST OF FIGURE ABBREVIATIONS

A	"A" band of striated muscle
ac	accessory cell
acf	actin filament
ap	attachment process, subcuticular muscle cell
c	coelom (mixocoel)
ca	canaliculus
cb	coelomic boundary layer
cc	cup-shaped cell
cd	central disc
co	collar
cp	cell process, modified subcuticular epidermal cell
d	desmosome (= <u>macula adherens</u>)
dc	dense cellular element
dex	deep exocuticular zone
e	extension of coelom
ec	subcuticular epidermal cell
en	endocuticle
enf	endocuticular fibril
ep	epicuticle
er	endoplasmic reticulum
ex	exocuticle
exf	exocuticular fibril
exp	exocuticular pore
fa	<u>fascia adherens</u>
G	Golgi complex

h	host, microenvironment of the parasite
I	"I" band of striated muscle
ic	intermediate cuticular zone
ids	infradiscal space
ij	intercellular junction
l	lamella of endocuticle
lb	lipid body
m	mitochondria
mec	modified subcuticular epidermal cell
mc	subcuticular muscle cell
mf	myofilament
mv	microvillus
myf	myosin filament
n	nucleus
nl	nucleolus
ns	neurosecretory granules
p	plasma membrane
r	free ribosome
sex	superficial exocuticular zone
sl	sarcolemma
sp	sarcoplasm
sr	sarcoplasmic reticulum
ss	subcuticular substance
tf	tonofibrils
T-s	"T"-system of striated muscle
v	vacuole
Z	"Z" band of striated muscle

Note: Unless specified otherwise in the appropriate figure legend, the section was stained as described in Chapter II. Abbreviations appearing on a plate are explained for at least one figure involved, but not necessarily for each one.

PLATE I

Figs. 1-4.--Cuticle and subcuticular cell layer of adults

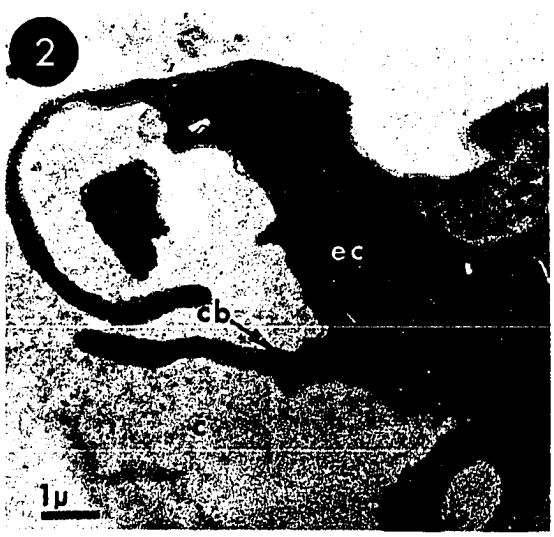
1.--The cuticle is divided into an extremely thin epicuticle (ep), a homogeneous exocuticle (ex) and a lamellate endocuticle (en). Subcuticular muscle cells (mc) and epidermal cells (ec) are flattened and their irregular shapes allow the coelom (c) to extend nearly to the endocuticle. The host microenvironment (h) for adults is the lumen of the lung. X4,400. Unstained section.

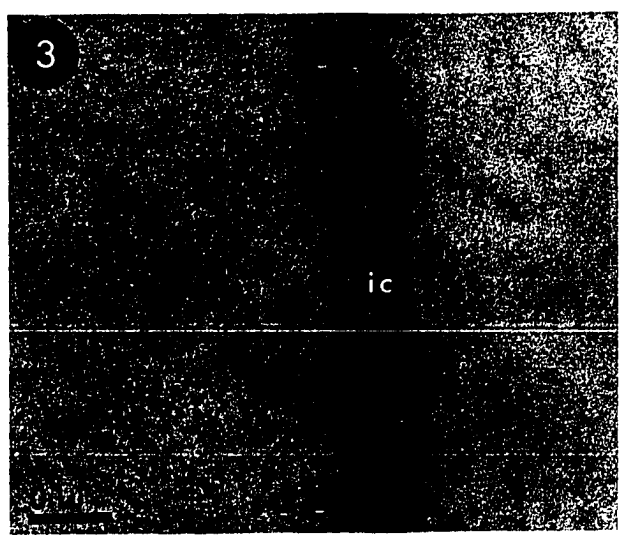
2.--Coelomic surfaces of all cells possess a coelomic boundary layer (cb). Subcuticular cells often show enhanced surface contact with the coelom (c) due to surface amplification. X7,300.

3.--Exocuticle (ex) is separated from endocuticle (en) by an electron dense intermediate cuticular zone (ic). X108,800.

4.--Inward extensions of the exocuticle (ex) bear no relationship to the attachment of subcuticular muscle cells (mc). X10,500.


1
ep
h
c
mc
ec


2
ec
cb
c
1μ


3
ic

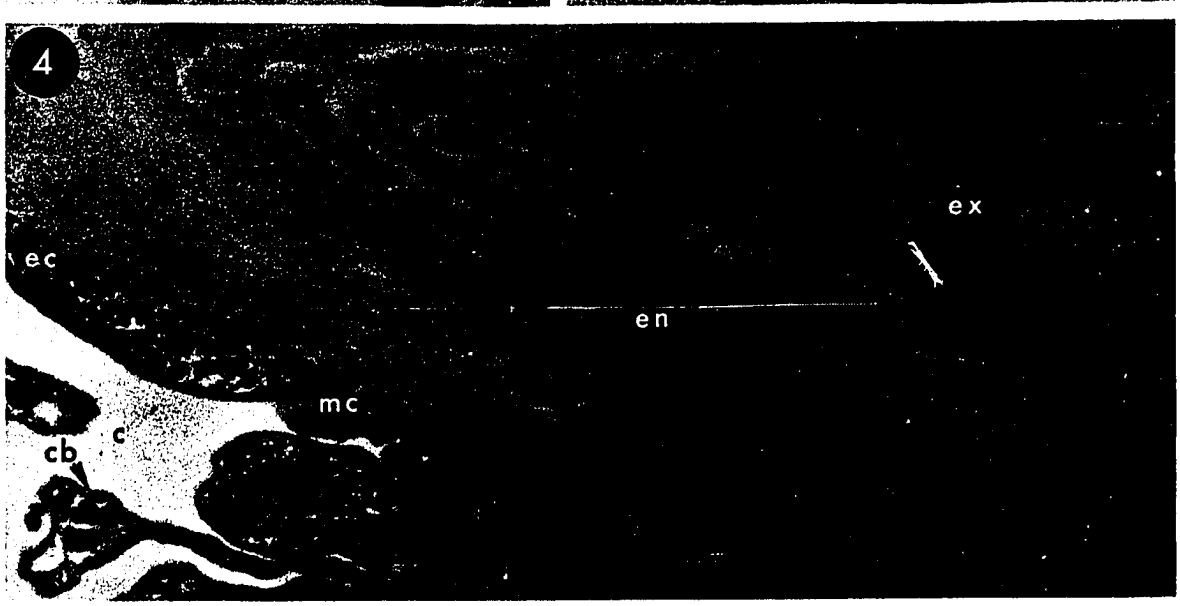

4
ex
ec
en
mc
c
cb

PLATE II

Fig. 5.--Subcuticular cell layer

5.--Intercellular junctions (ij) include specialized maculae adherentes (= desmosomes) (d) and septate fasciae adherentes (fa). Attachment processes (ap) of the subcuticular muscle cells (mc) connect myofilaments (mf) to the endocuticle (en) by means of tonofibrils (tf) which intertwine with the endocuticular fibrils. Mitochondria (m) are present in both subcuticular muscle cells (mc) and epidermal cells (ec). The latter show rough ER (er) and apparent free ribosomes (r). Cuticular cell surfaces of both epidermal and muscle cells show microvillar (mv) specializations. All cell surfaces exposed to the coelom (c) are covered with the coelomic boundary layer (cb). Occasional canaliculi (ca) extend between the subcuticular cells. X26,100. Section stained with uranyl acetate alone.

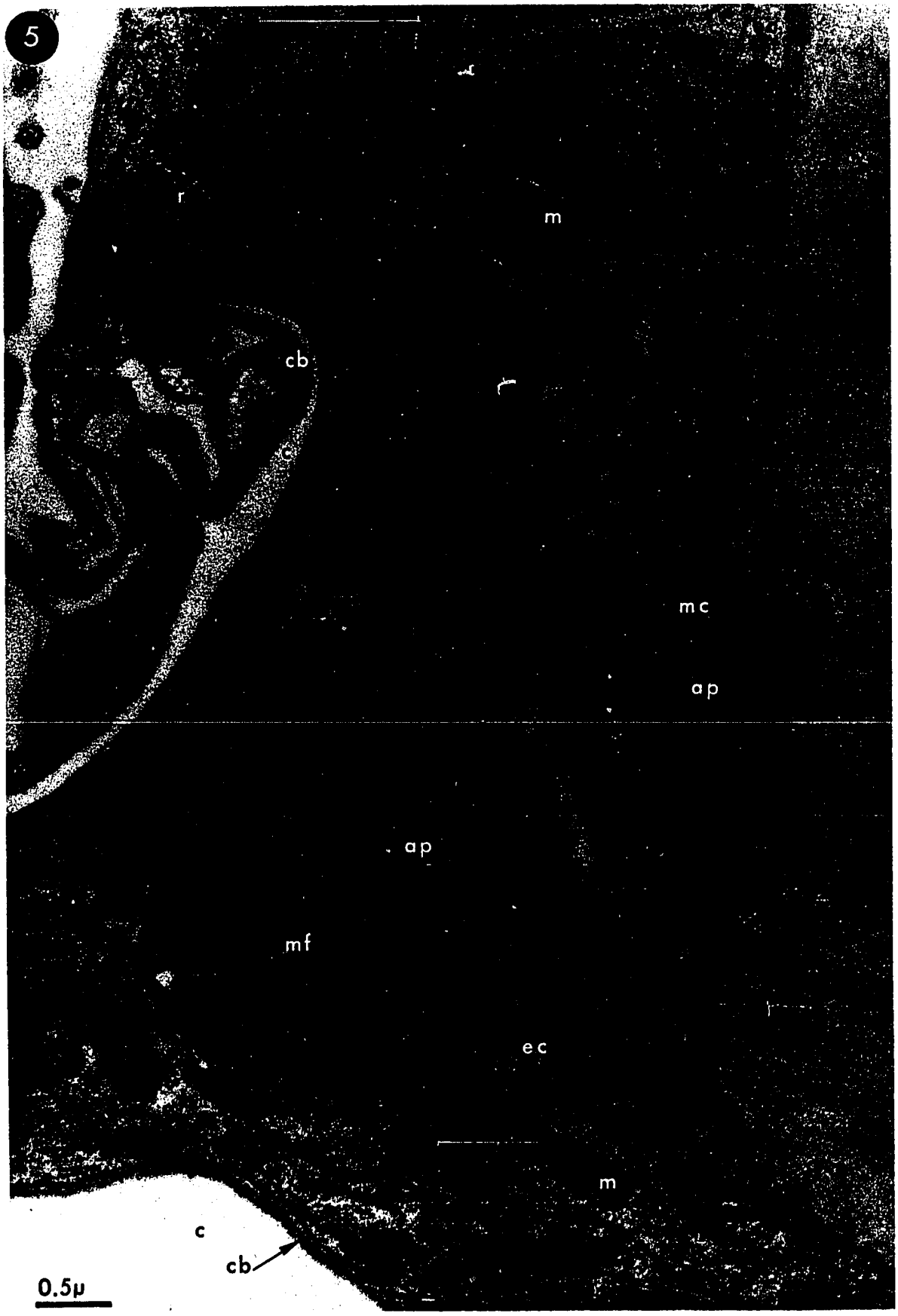

5
r
m
cb
mc
ap
ap
mf
ec
m
c
cb
0.5µ

PLATE III

Figs. 6-9.--Details of microvilli, the epi- exocuticlar relationships in adults

6.--Microvilli (mv) are recumbent, surrounded by endocuticle (en) and appear bulbous. Note the desmosome (d) in the intercellular junction (ij). X145,300.

7.--The epicuticle (ep) is a thin dark line lying between the thin, less dense mucus or wax layer and the complex exocuticle (ex). Exocuticular fibrils (exf) appear in various sections as straight lines, parabolas, or cross-sectional spots. The exocuticle shows different kinds of bundling and ordering of the exocuticular fibrils in the superficial exocuticular zone (sex) and the deep exocuticular zone (dex). Host area (h) is the lumen of the lung. X85,000.

8.--Electron-dense cords of regular form occur in the superficial exocuticular zone. Note the absence of the wax layer over the epicuticle (ep). X66,000.

9.--Electron-dense cords also occur in irregular forms. A wax layer is present over the epicuticle (ep). X71,000.

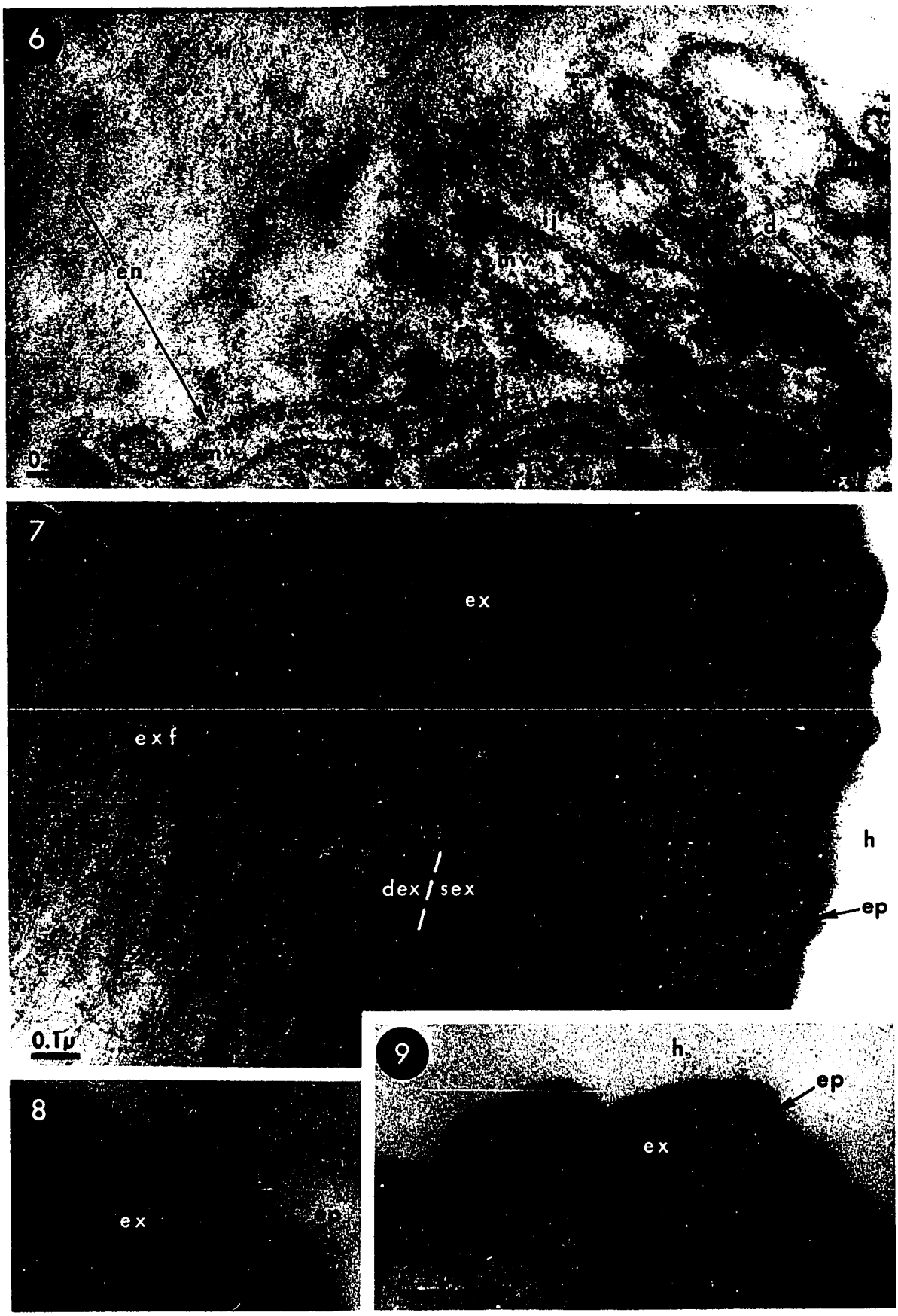

6
en
inv
d
0.1μ
7
ex
exf
dex / sex
h
ep
0.1μ
8
ex
9
h
ep
ex

PLATE IV

Figs. 10-13.--Details of the endocuticle and subcuticular cell layer in adults

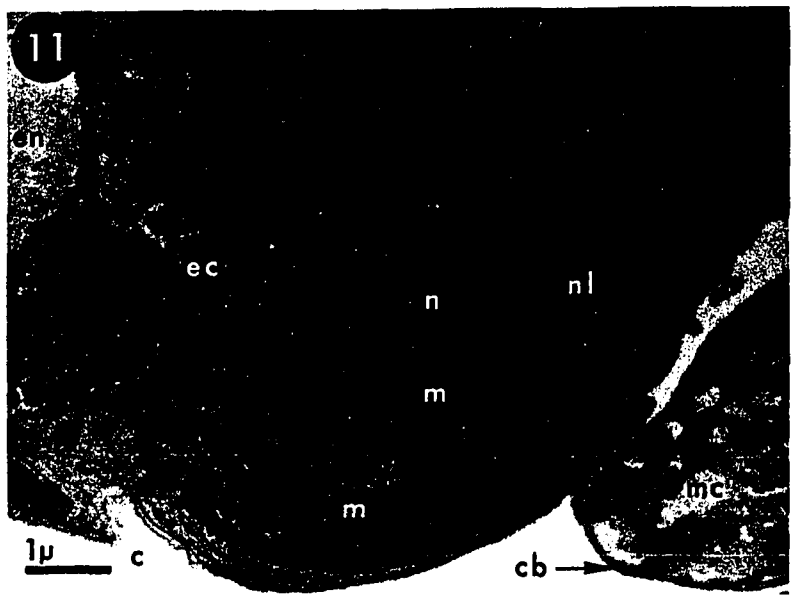
10.--Two intercellular junctions (ij) illustrate the lack of specialized junctions at the coelomic side of the body wall. X52,700.

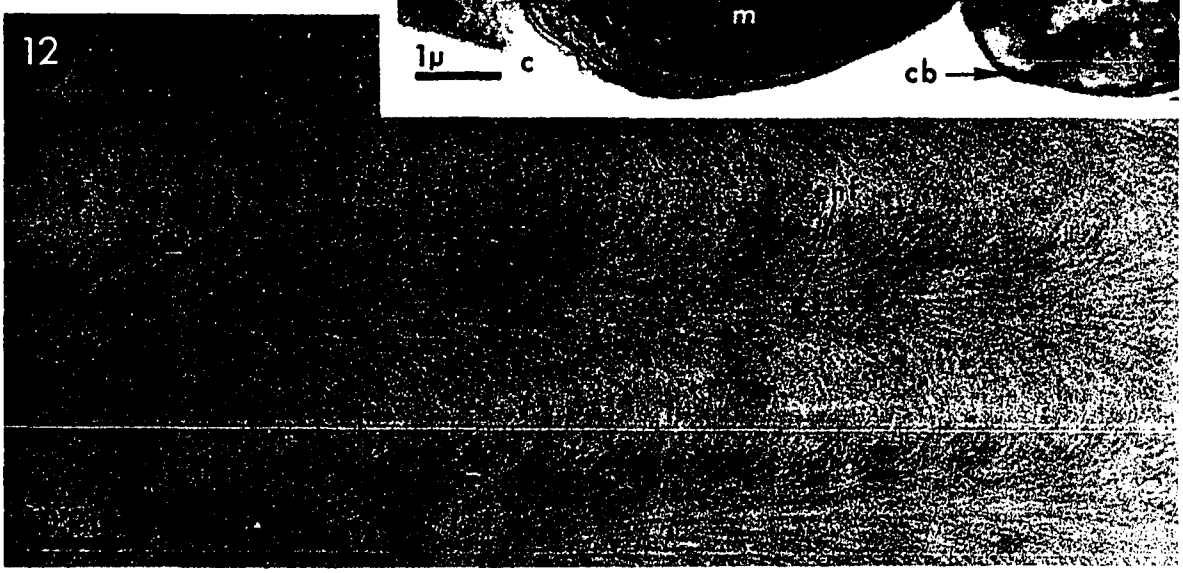
11.--Apparent mitochondrial (m) fusion is observed. Uninucleolate (nl) nuclei (n) are rarely seen in the attenuated subcuticular epidermal cells (ec). The coelomic boundary layer (cb) blankets the coelomic surface of muscle cells (mc) in addition to the subcuticular layer of cells. X11,400.

12.--The lamellar (l) appearance of the endocuticle (en) results from parabolic orientation of endocuticular fibrils (enf). The lamellae are roughly parallel to the cuticular surface. X72,600.

13.--Near the subcuticular cells the endocuticle (en) is less well organized into lamellae. Endocuticular fibrils (enf) occur in arranged groups present in both cross-sectional (arrow 1) and longitudinal (arrow 2) profiles. The microvilli or short cell processes (cp) characterize a very early stage in the molting process. Note the dark material at the inner edge of the endocuticle (arrow 3) and the occurrence of microtubules (arrow 4), and vacuoles (v), all characteristics of molting. X55,200.


10
jj
cb
c


11
ec
n
nl
m
m
mc
1μ
c
cb


12
jj

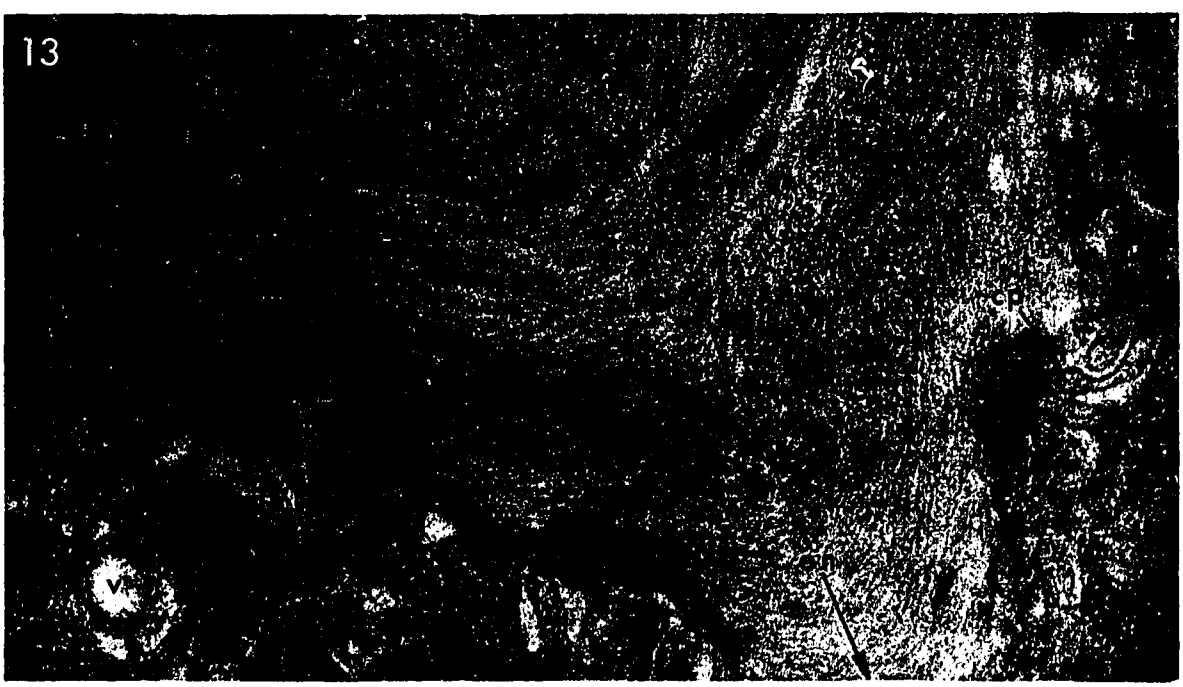
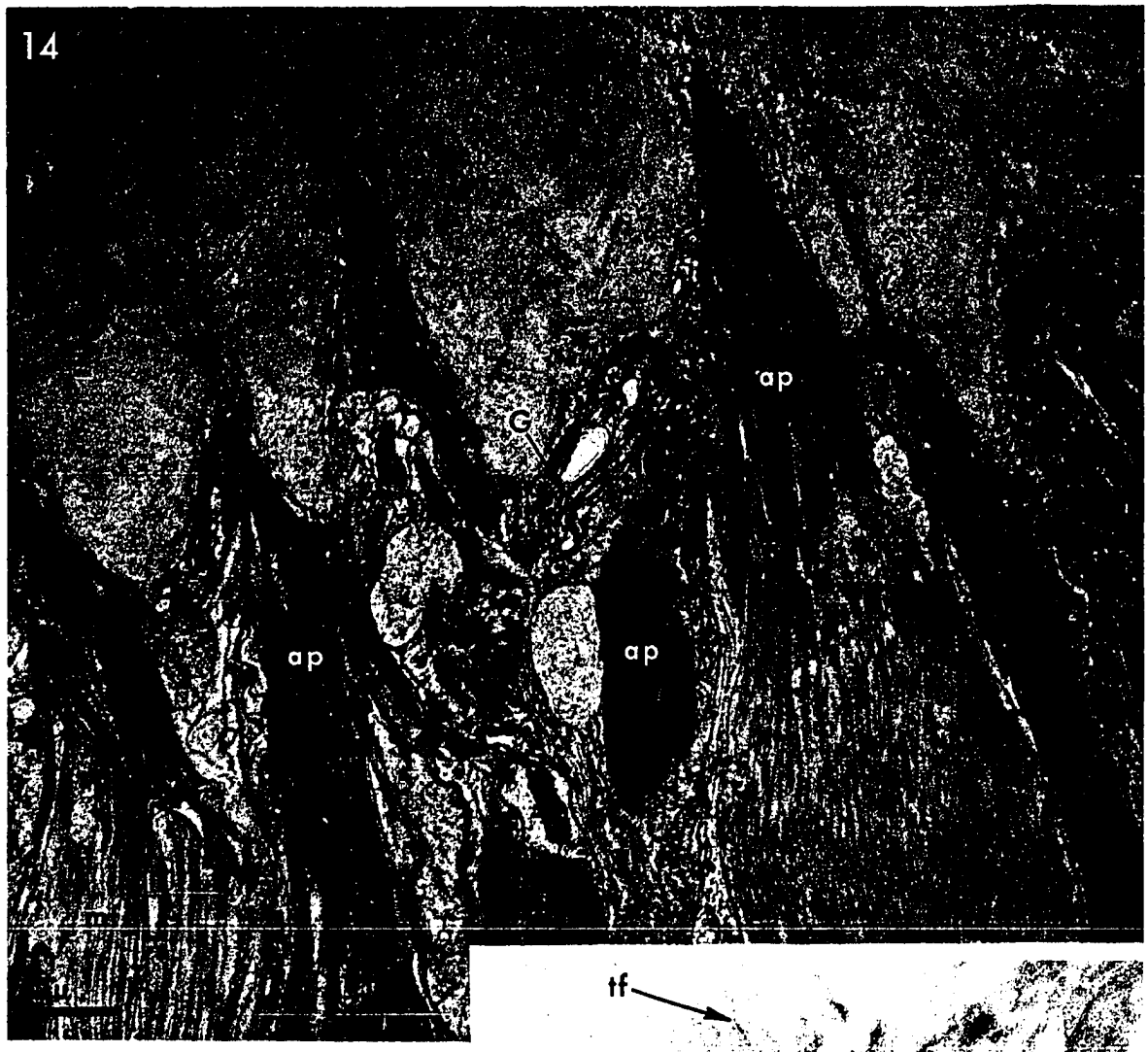

13
v

PLATE V

Figs. 14 and 15.--Subcuticular muscle cells in adults

14.--The muscle cell (mc) insertion into the endocuticle (en) is comprised of attachment processes (ap) consisting of dense bundles of tonofibrils (tf) extending from the myofilaments (mf). Golgi complexes (G) are seen in the muscle cells. X6,100.

15.--Intercellular junctions between muscle cells (mc) and epidermal cells (ec) include maculae adherentes (d) and septate fasciae adherentes (fa). The microvillar (mv) arrangement of the cuticular cell surface is typical of the intermolt situation. Both cells contain mitochondria (m) and a lipid body (lb) is in the muscle cell. X33,200.


14
ap
G
ap
ap

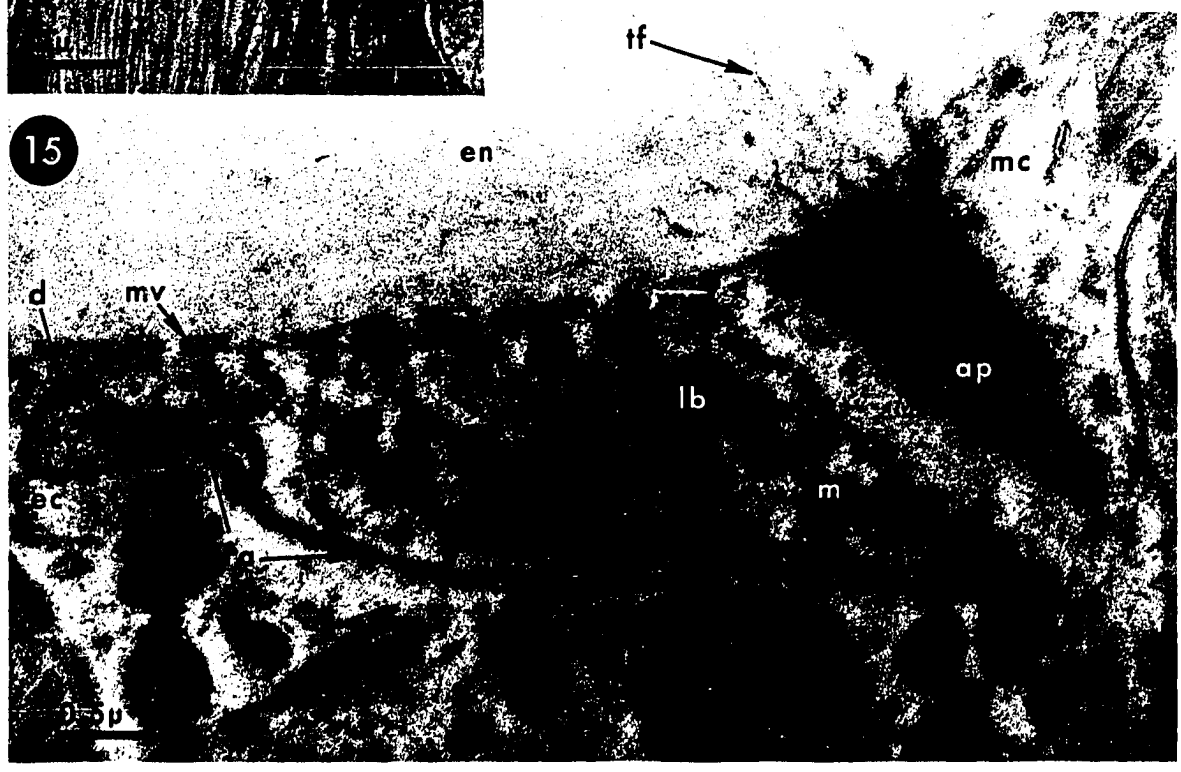

15
tf
en
mc
d
mv
ap
lb
m
ec
g
0.5 µ

PLATE VI

Figs. 16-20.--Details of muscle cells and their attachment processes in adults

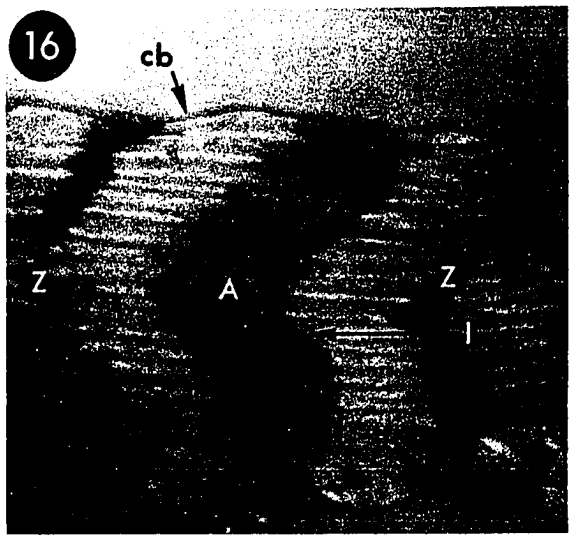
16.--Muscle cells show vertebrate-type striations, only "A," "I" and "Z" bands being visible. X6,600. Unstained section.

17.--In cross-section, typical arrangements of actin (acf) and myosin (myf) filaments are visible. The sarcolemma (sl) is invested with coelomic boundary layer (cb). X96,500.

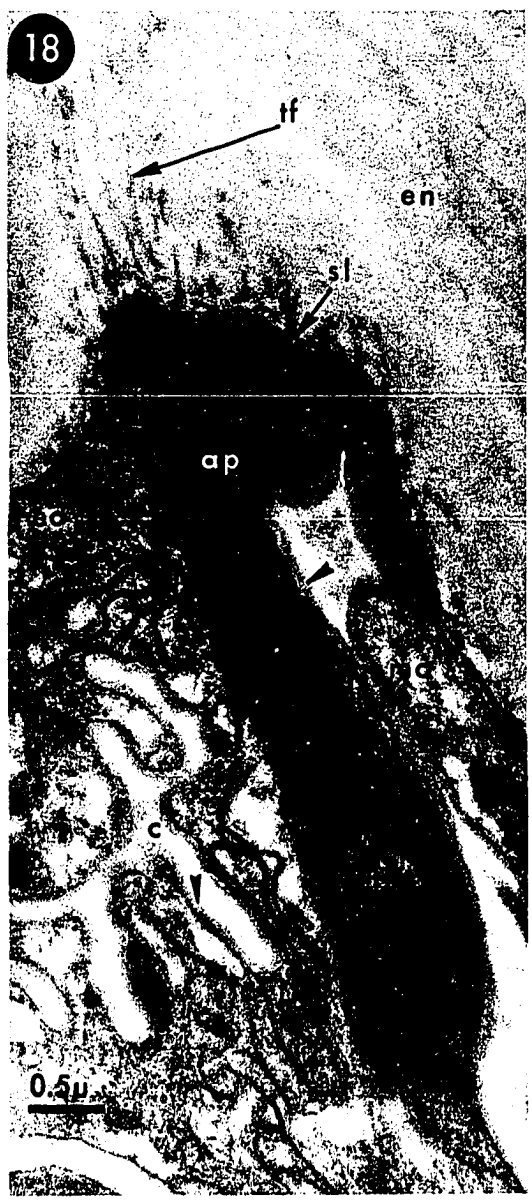
18.--Both muscle (mc) and epidermal (ec) cells have elaborate coelomic (c) surfaces blanketed by the coelomic boundary layer (arrowheads). The sarcolemma (sl) of the muscle cell (mc) is modified into involuted cones at the endocuticular (en) surface of the attachment process (ap). Tonofibrils (tf) extend from the attachment process through these cones into the endocuticle. X21,100.

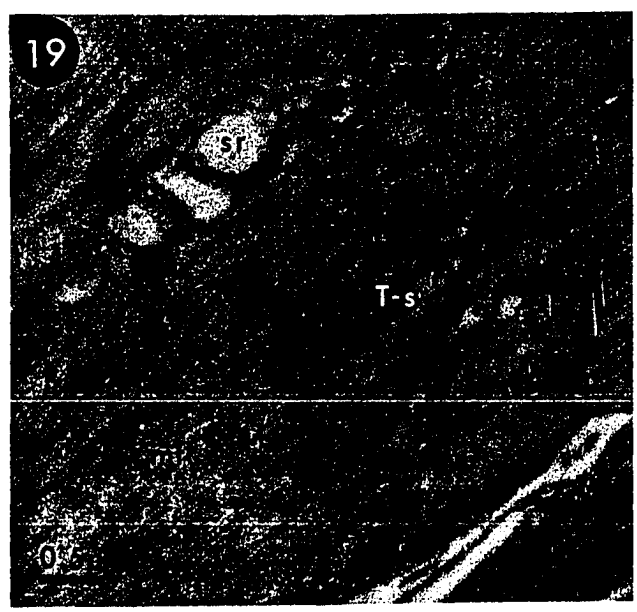
19.--Muscle cells (mc) show the sarcoplasmic reticula (sr) and T-system (T-s) typical of striated muscle. X17,400.

20.--Involuted cones of the sarcolemma are seen here in cross section. X63,000.


16
cb
Z
A
Z
I


17
c


18
tf
en
sl
ap
c
c
c
0.5μ


19
sl
T-s
0

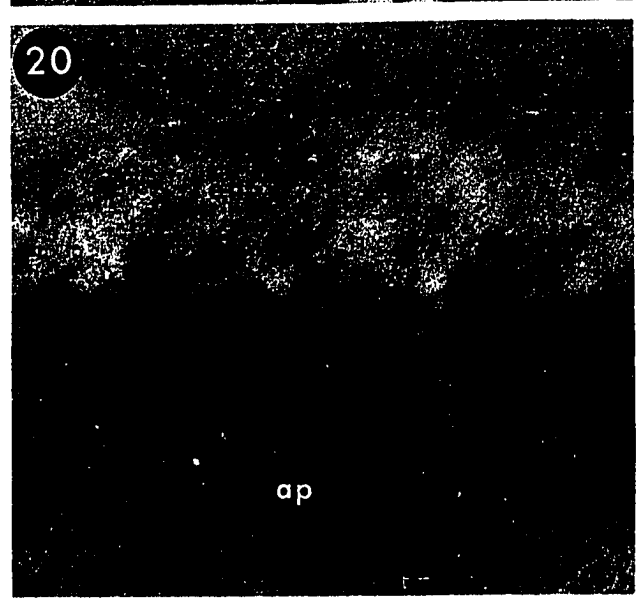

20
ap

PLATE VII

Figs. 21-26.--Muscle cell attachment processes and cuticular molting in adults

21.--Discontinuities (arrows) in the dense "Z" band indicate continuity between myofilaments (mf) and the attachment process (ap). X26,000. Section stained with uranyl acetate alone.

22.--In early stages of molting, the muscle insertions are intact with tonofibrils (tf) extending across the subcuticular molting space (ss). Vacuole (v) formation is common in subcuticular cells during molting. X16,700.

23 through 26.--Sequential stages in molting show the progressive elaboration of labyrinthine, ordered cell processes (cp), and the progressive increase subcuticular substance (ss) and resultant separation of the endocuticle (en) from the underlying cells. The number of free ribosomes (r) increases as well. Fig. 23, X87,300; Figs. 24 through 26, X16,700.

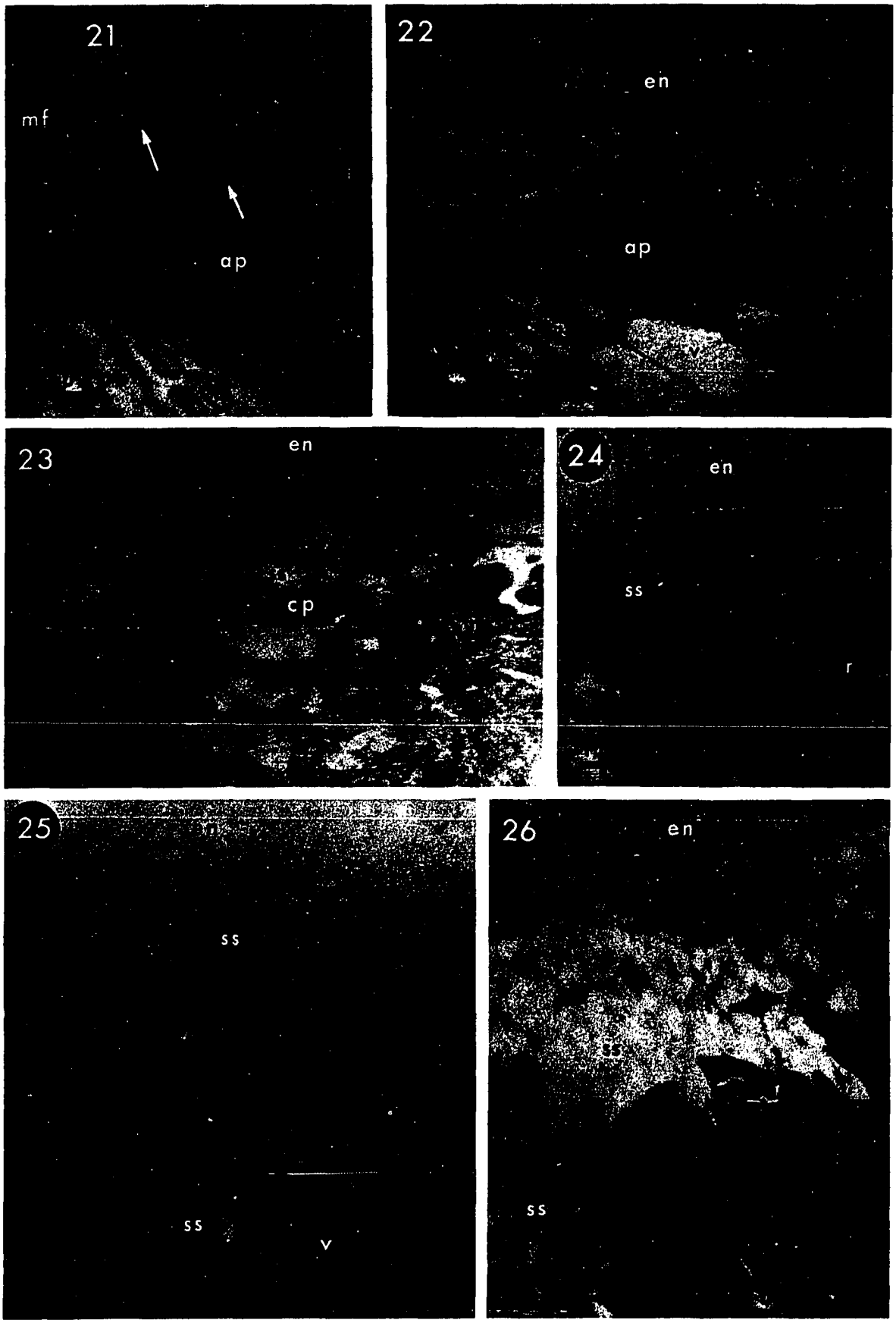

21
mf
ap
22
en
ap
v
23
en
cp
24
en
ss
r
25
ss
ss
v
26
en
ss
ss

PLATE VIII

Fig. 27.--Infective nymph body wall

27.--Like that of adults, infective nymph cuticle is composed of epicuticle, exocuticle (ex) and endocuticle (en). The exocuticle and endocuticle are separated by an intermediate cuticular zone (ic). Nymph cuticle is in intimate association with host tissue (h) (in this case, liver). The subcuticular epidermal cells (ec) are generally pleomorphic, particularly in their basal portions (just visible at the bottom of the figure). The principal mass of the cells (i.e. adjoining the cuticle) presents an orderly columnar profile with little interdigitation with adjacent cells. The epidermal cells are rich in mitochondria (m), are uninucleate (n), and possess Golgi complexes (G) as well as smooth ER (er) in the area of the nucleus. Microvilli (mv) occur near the periphery of the cuticular surface of epidermal cells during the intermolt phase. X5,500.

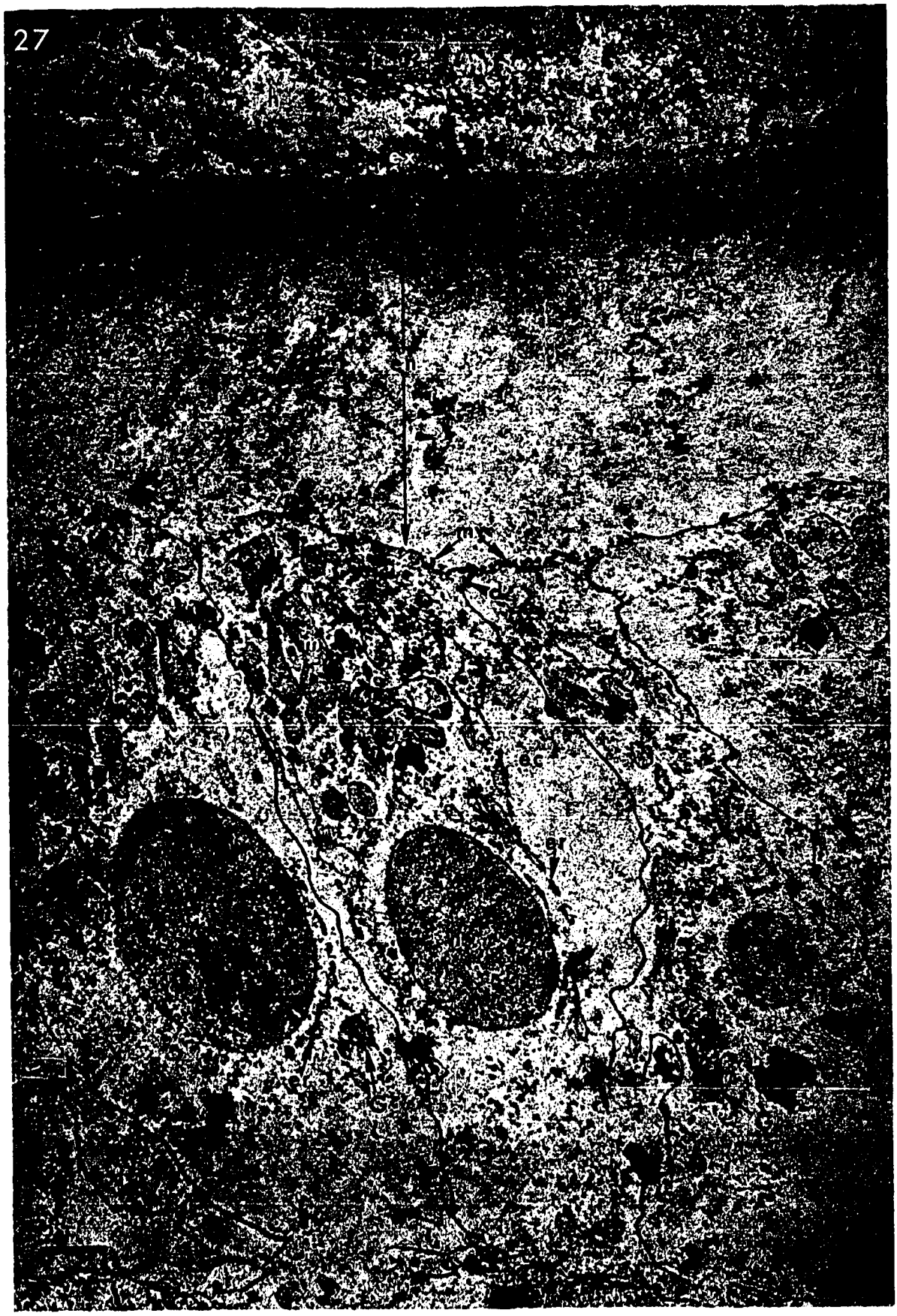

27
ex
m
ec
er

PLATE IX

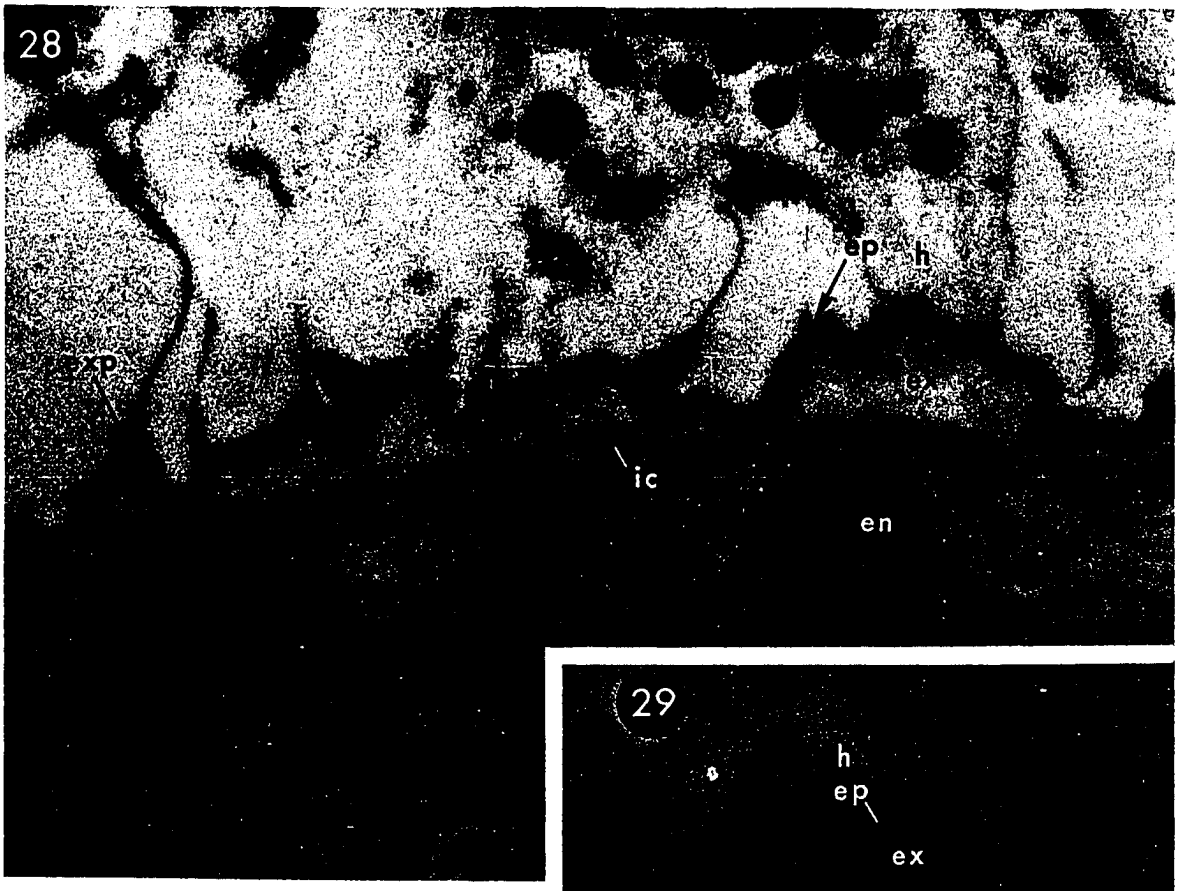
Figs. 28-31.--General nymph cuticle and a sensory specialization of the subcuticular cells

28.--Exocuticular (ex) material is extruded through exocuticular pores (exp) in the form of filaments that extend out to host tissue (h). Fibrils are present in the endocuticle (en), but are oriented as waves rather than parabolas. X15,800.

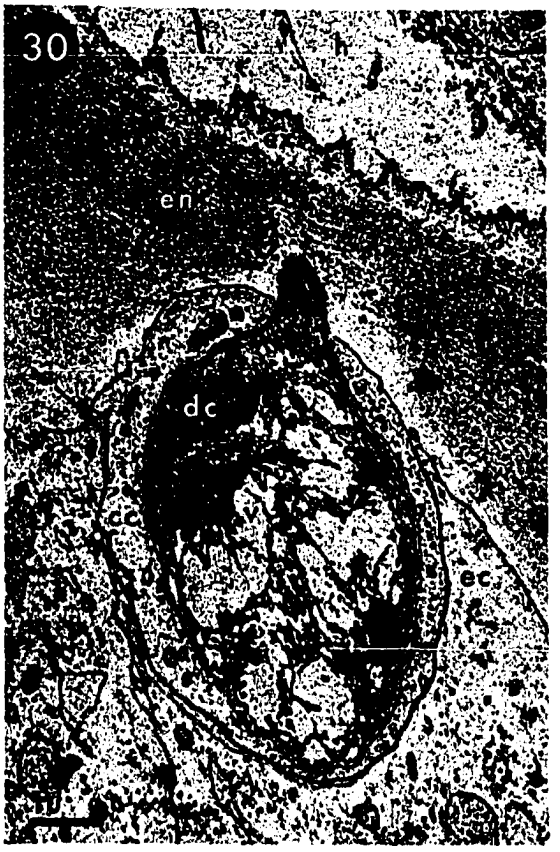
29.--Spines composed of epicuticle (ep) and exocuticle (ex) deform adjacent host liver cells (h). X21,700.

30.--Nymphs possess a cup-shaped cell (cc) in the epidermal layer that surrounds a teardrop shaped dense cellular element (dc). Desmosomes (d) attach the cup-shaped cell to the epidermal cells (ec) but not to the dense element. No known organelles are identifiable in the latter element. X9,700.

31.--The dense cellular element (dc) has a distinct trilaminar plasma membrane (p) that is separated from the cup-shaped cell by dense amorphous material that is contiguous with the endocuticle (en). The cup-shaped cell contains mitochondria (m) and small vesicles that may be neurosecretory granules (ns). X23,100.


28
ep
h
xp
ic
en

29
h
ep
ex
ic
en


30
h
h
en
dc
ec
ec

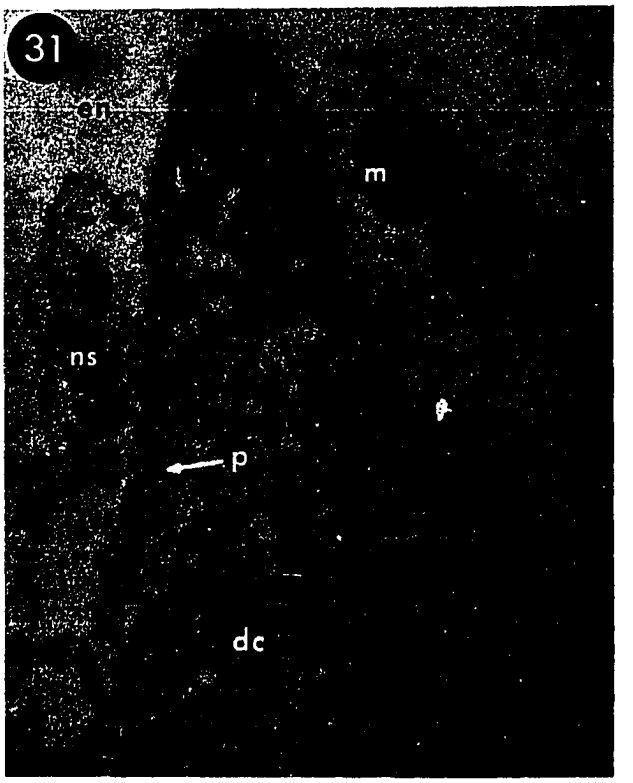

31
en
m
ns
p
dc

PLATE X

Figs. 32-36.--"Stigmata" in nymphs

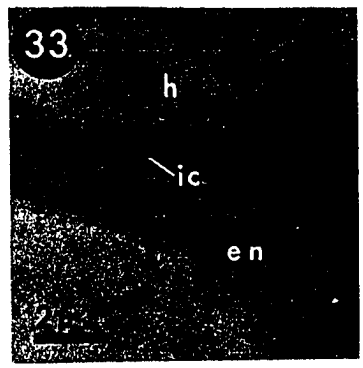
32.--The "stigmata" consist of two uninucleate (n) modified epidermal cells (mec) and associated accessory cells (ac) related to an overlying modified portion of the cuticle. The cells are joined by smooth intercellular junctions (ij). The cuticular structure is composed of a circular central disc (cd) surrounded by a peripheral collar (co). Host tissue (h) is liver. Material in the infradiscal space (ids) completely fills the area between the cell processes (cp). X6,300.

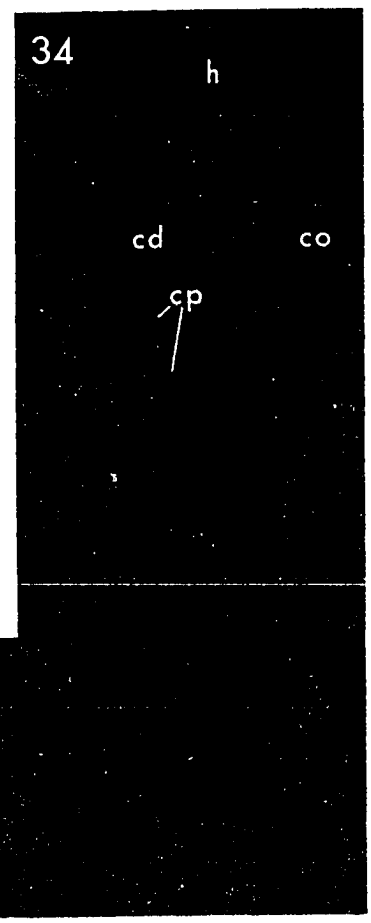
33.--A tangential section normal to but also peripheral to the collar of a "stigmata." X4,800.

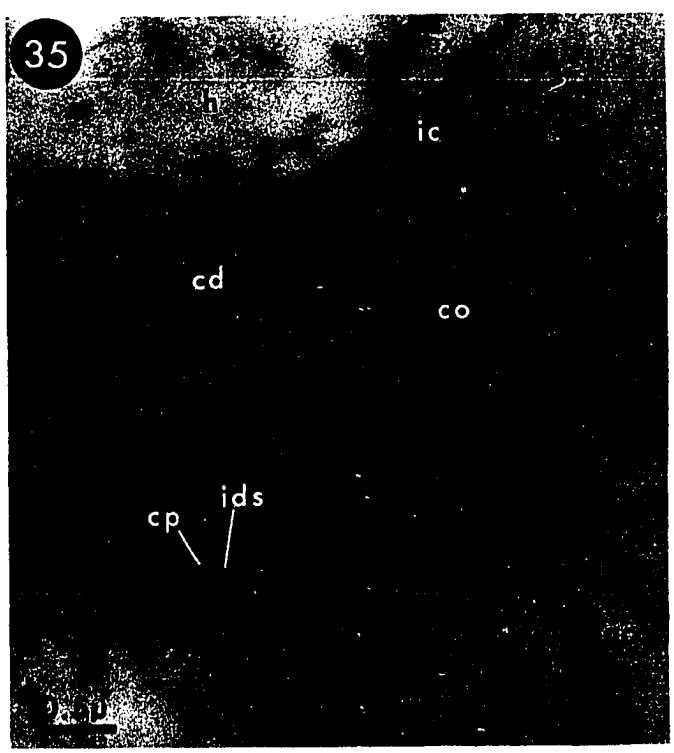
34.--The modified epidermal cell exhibits cylindric cell processes (cp) at its distal surface which extend to the base of the central disc (cd). X14,600.

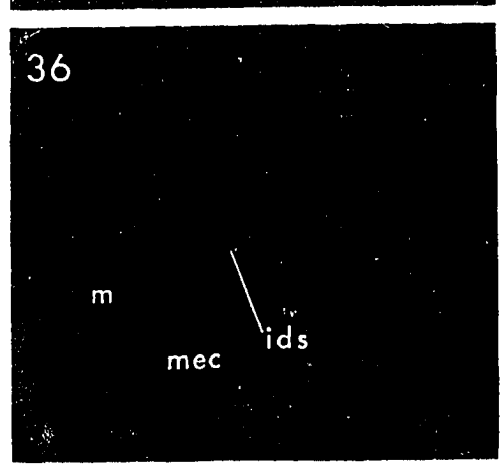
35.--The detailed ultrastructural relationships of the cell processes (cp) and the infradiscal space (ids) are shown at higher magnification here. The central disc is structurally identical to and continuous with the exocuticle (ex). The collar of the "stigmata" is structurally continuous with the intermediate cuticular (ic) zone and mirrors its spacial relationships with the exocuticle (ex) and the endocuticle (en). Thus, its homology with the intermediate cuticular zone is indicated both inside and outside the "stigmata" complex. X19,900.


32
ic
h
en
co
co
m
mec
n


33
h
ic
en


34
h
cd
co
cp


35
h
ic
cd
co
cp
ids


36
m
ids
mec

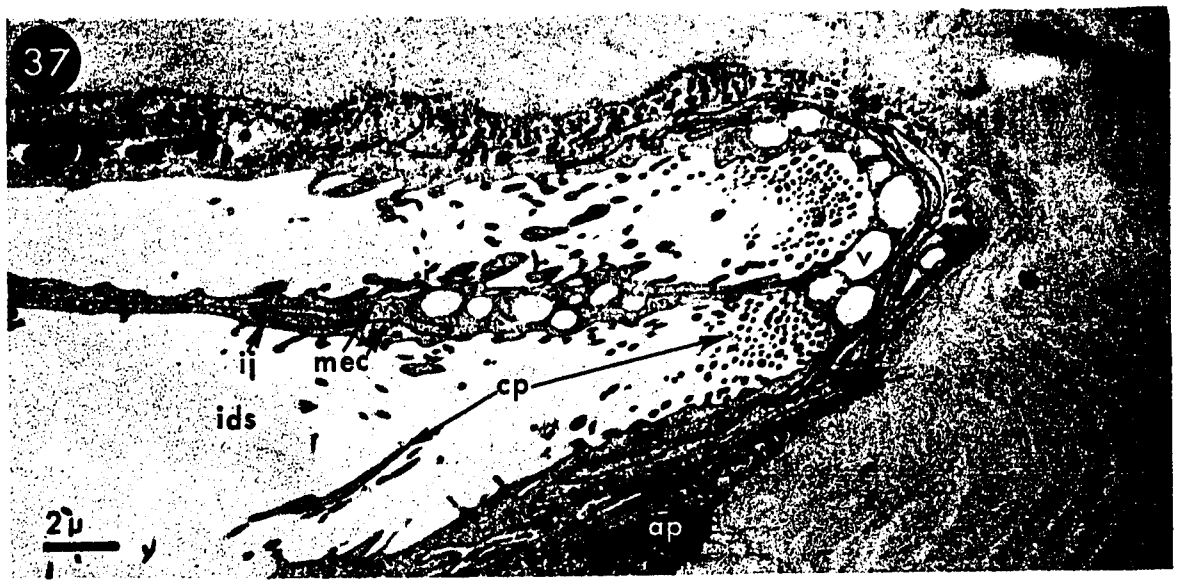
36.--The modified epidermal cells (mec) contain mitochondria (m) near the bases of the cell processes. X11,900.

PLATE XI

Figs. 37 and 38.--"Stigmata" in adults

37.--In adults the "stigmata" have the same basic structure as in the nymphs. The section here is essentially a transverse profile through the "stigmata" at the level of the infradiscal (ids) region showing infradiscal substance and cell processes (cp) of the modified epidermal cells (mec). The intercellular junction (ij) of the two modified epidermal cells shows interdigitation. Cytoplasm of these cells is highly vacuolated (v). The insertion of a muscle cell (mc) into the endocuticle (en) is seen at the attachment process (ap). X5,100.

38.--At the level of origin of the cell processes a transverse profile shows the infradiscal space is filled with the cell processes (cp). Extensions (e) of the coelom (c) penetrate the "stigmata" complex and terminate close to the infradiscal space (ids). X10,500.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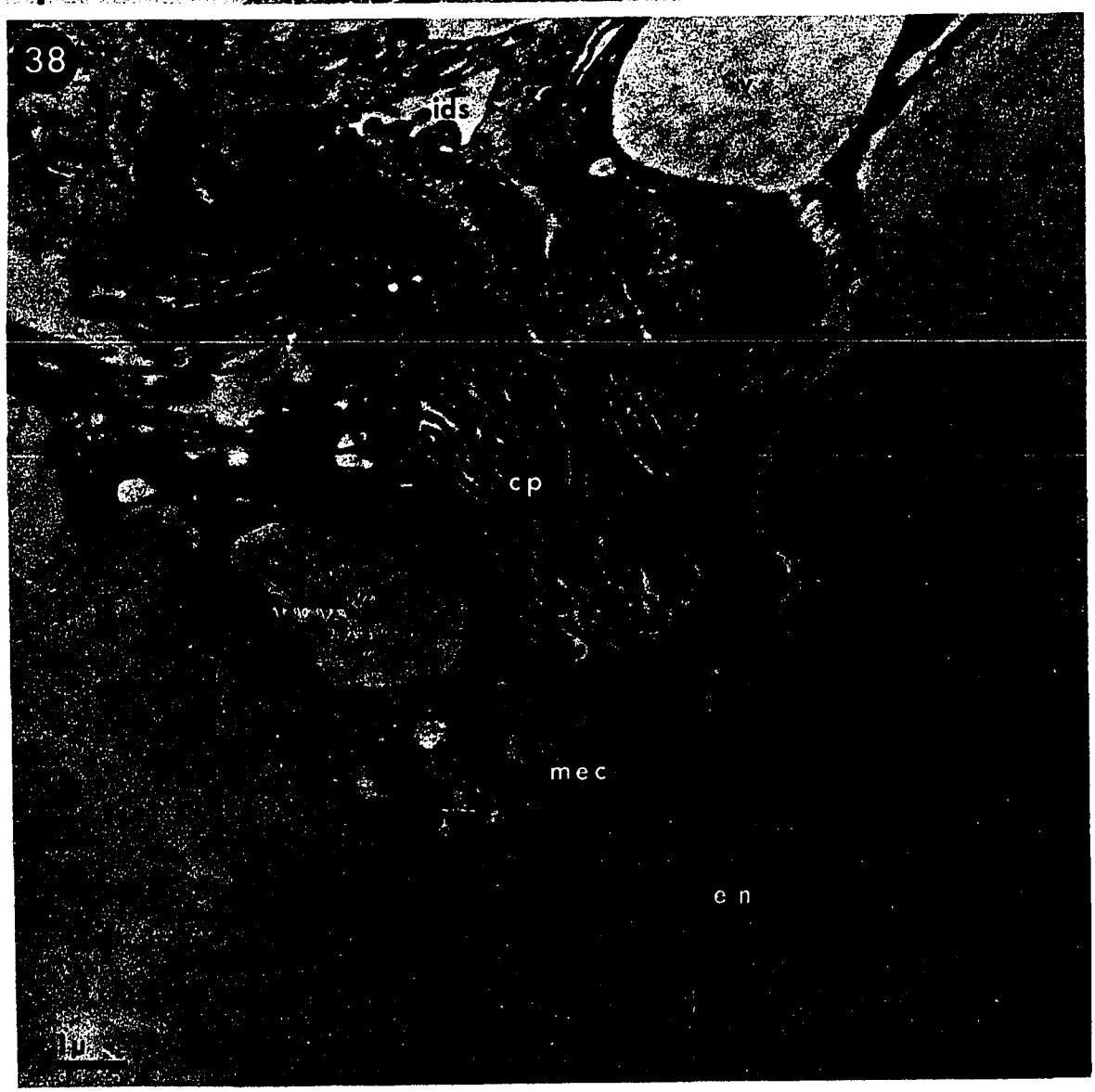
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CHAPTER IV

DISCUSSION

Pentastomids can be called helminths only under a broad definition such as that of Chandler and Read (1961), who defined helminths (or worms) as "any elongate creeping thing that is not obviously something else."

The pentastomid cuticle is basically a simplified arthropodan cuticle and, as such, is unlike that of any other endoparasitic helminth. It is most like the external surfaces of nematodes (Chitwood and Chitwood, 1950) and acanthocephalans (Crompton and Lee, 1965) in that it is a true cuticle and not a cytoplasmic tegument.

Integuments of all organisms are actually organ systems of extreme importance with a variety of functions. The significance of the system is increased when the organism is endoparasitic. The integument is perhaps most highly evolved in the Cestoda and Acanthocephala where it functions as an absorptive surface comparable to a gut lining in addition to its protective and sensory roles. The cuticle in both the Arthropoda and the Pentastomida functions as skin, as skeleton, and, at specialized areas, as a stimulus receptor.

Electron microscopy has been increasingly used in the study of endoparasitic helminth integuments. Lee (1966) provides an excellent review of the integuments of acanthocephalans, cestodes, nematodes and trematodes. Ultrastructural analyses of free living invertebrates have become common as well. Recent descriptions of the integuments of suspected pentastome relatives include those of the Annelida (Millard and Rudall, 1960; Laverack, 1963), the Tardigrada (Crowe et al., 1970; Baccetti and Rosati, 1971) and the Onycophora (Robson, 1964).

Arthropod cuticles have been the subject of numerous research publications. Summaries of the structural aspects of various cuticles are given by Dennell (1960), Locke (1964), Noble-Nesbitt (1968), Richards (1951, 1953) and Wigglesworth (1948b, 1957, 1965).

The typical arthropod cuticle, like that of P. crotali, is composed of three layers, the non-chitinous epicuticle and the chitinous exo- and endocuticles.

It is immediately apparent from electron micrographs that the cuticle of P. crotali is a solid protective layer. It is not known whether the cuticle is completely impermeable, no uptake studies having been done, but it is doubtful that any nutritionally significant uptake could occur through this layer. The impervious appearance further implies that absorption and excretion both occur through the gut wall.

On the basis of the dimensions given, it is apparent that the "exocuticle" described by Doucet (1965), and Hett (1924) as well as the "epicuticle" of Osche (1963) and Richards (1951) include both the epicuticle and exocuticle as described above. The thinness of the epicuticle and the fact that the epicuticle and exocuticle stick together in light microscopy preparations explains the prior interpretation given these layers. These two layers often break away from the endocuticle as a single layer when sectioned for light microscopy.

The epicuticle reported here may be the thinnest known for any arthropod. The thinnest with which the author is familiar is the 200 to 300 A epicuticle of the collembolan Podura aquatica (Noble-Nesbitt, 1963).

The typical insect epicuticle is quadripartite (Locke, 1964). The layer observed in P. crotali appears to be homologous with the proteinaceous layer termed "cuticulin" by Wigglesworth (1934). The cuticulin layer itself has been reported to be triply subdivided (Nobel-Nesbit, 1968) in insects although it is apparently homogenous in P. crotali. The thickness reported here corresponds closely in adults with that for Podura aquatica, although the epicuticle in P. crotali nymphs is slightly thicker.

The superficial layer occasionally seen in adults (Figs. 7 and 9) is of questionable origin and function. It corresponds in location to the wax layer of insects (Locke,

1964). It may, for a number of reasons, be identical to the layer of host mucus described by Doucet (1965). First, it is only visible in certain sections, as was his layer. Second, in insects this layer is deposited after the cuticle is formed via dermal glands and pore canals. Both these are absent in the cuticle here. Finally, the wax layer functions as a complex water barrier in insects (Locke, 1964). If this were a wax layer it would seem that the cuticle would demonstrate a higher degree of hydrophobia.

Whatever its true nature, it was evidently thick enough to be resolved in the specimens examined by Hett (1924) and Heymons (1935).

In contrast to the epicuticle, the portion of the cuticle containing chitin (in association with proteins) is collectively referred to as the procuticle. In the majority of arthropods it is differentiated into an outer exocuticle and an inner endocuticle.

The presence of chitin has long (Schulze, 1932) been used as a strong point in the argument for arthropodan affinities of the pentastomes. While this is a valid point--and one confirmed by this study--it should be noted that the Annelida, Coelentrata, Mollusca, and Brachiopoda all contain the B-form of chitin in restricted areas of their cuticles (Rudall, 1955). a-chitin is the only form present in (all) arthropods (Dennell, 1960). X-ray characterization of the chitin in a pentastomid would strengthen this point still further.

The exocuticle is that portion of the cuticle that usually imparts hardness and rigidity to the cuticle (Noble-Nesbitt, 1968). This is the result of quinone tanning of the proteins of this layer (Blower, 1950; Cottrell, 1964).

The lack of general cuticular tanning in P. crotali is evident in its color, transparency, and elasticity. This may reflect a difference between the exocuticle of pentastomes and arthropods. Tanning does occur in the modified cuticle of the hooks of pentastomes.

It is difficult to attach any adaptive significance to the lack of tanning in pentastome cuticles. The lack of extensive locomotion as required by free-living arthropods at least explains how P. crotali has survived without it. Hydrostatic tension of the coelomic fluid must provide support necessary for the exoskeletal role of the cuticle.

The presence of spines on the nymphal exocuticle is probably of adaptive significance as locomotion is required during these migrating stages.

The intermediate cuticular zone of the nymphs and adults is in the same position as the mesocuticle reported from a few insects. It is not lamellate as reported from the thysanuran Thermobia (Noble-Nesbitt, 1968) and the layers are probably not homologous. While I have not considered it a distinct layer (rather, merely a separating zone) of the cuticle, to do so would make the cuticle in P. crotali tetrapartite.

In pentastomes, as in soft-bodied insects (Noble-Nesbitt, 1968), the endocuticle comprises the major portion of the cuticle. As in insects (Locke, 1964) and all other arthropods (Richards, 1951), the endocuticle of P. crotali is composed of lamellae.

The formation and significance of these lamellae are reviewed by Locke (1964) and Neville (1967). The completeness of the fibrils (Fig. 12) would seem to preclude the suggestion of Bouligand (1965) that the parabolic shapes of the chitin fibrils are optical illusions resulting from Moiré patterns. My results are not conclusive enough to refute the theory, however. As suggested by Drach (1953) and Slifer and Sekhon (1963), the plane of the parabolas may well not be in a plane perpendicular to the cuticular surface.

It is known that the endocuticular fibrils are chitin and the electron-lucid matrix is proteinaceous (Neville, 1967). The nature of the matrix is unknown in P. crotali, although it may be related to the protein complex termed "resilin" (Andersen and Weis-Fogh, 1964), since both are elastic and transparent. Resilin has been reported only in specialized areas of arthropod cuticle. Its occurrence in the entire cuticle, if proven, would be noteworthy.

In P. crotali it appears that lamellar formation is at least begun adjacent to the epidermal cells as suggested by Locke (1961).

Circadian rhythms in the environment of insects have been linked to lamellar formation (Neville, 1967). The lack

of lamellae in nymphs may be an indication of a more homeostatic microenvironment for nymphs than for adults. Such a difference is not surprising when the life cycle is considered.

Three categories of cells were seen in the epidermal layer: epidermal cells, muscle cells, and two types of modified epidermal cells. No oenocytes nor dermal glands as reported from insects (Wigglesworth, 1934) were present.

Richards (1951) states that arthropod epidermal cells may be polygonal (as seen here in nymphs) or syncitial (as may exist in the adults). He also indicates that vacuoles and other organelles reported here are present in the Arthropoda.

Striking differences were observed in the surfaces of the cells, depending on which of three different cellular microenvironments the particular surface was facing. The three different "environments" are the coelomic surface, the cuticular surface, and the junctions with other cells.

At the coelomic surface, the plasma membranes are smooth and unspecialized, and no cytoplasmic modifications are present.

The coelomic boundary layer in adults may function as a supportive "basement membrane," but its universal occurrence on the coelomic surfaces of muscle bundles, reproductive tracts, and gut wall preclude its being defined as such.

The lack of a basement membrane is previously reported for certain collembola (Noble-Nesbitt, 1963). The basement membrane reported by Richards (1951) in the pentastome Kiricephalus appears to be muscular tissue. Its absence in P. crotali may be explained by the necessity of the coelom to function as the circulatory system.

When facing other cells, two modifications of the standard or smooth intercellular junction were observed. In addition to maculae adherentes, fasciae adherentes of a specialized type occurred in the adults. Only the former occurred in nymphs.

The specialized fasciae adherentes appear to be enlarged septate desmosomes as described originally in Hydra (Wood, 1959). Apparent tonofibrillar concentrations occur in the cytoplasm adjacent to the fasciae as they do in desmosomes. The transverse bars appear to be joined to the adjacent plasma membranes in certain areas as suggested by Wood (1959). I propose the term "septate fasciae adherentes" for these structures.

A similar structure is reported from the annelid Lumbricus (Coggeshall, 1966). He notes that, unlike desmosomes, these structures may be curved (as in P. crotali), and that the transverse septa may be ridge-like, encircling the cell.

These intermediate junctions may have structural significance in P. crotali in light of the elasticity of the cuticle and the lack of a basement membrane.

Cell surfaces abutting the endocuticle show different features from those facing other cells or the mixocoel, and these features undergo changes during molting as described above.

During intermolt, the subcuticular cell surfaces are microvillate and the plasma membranes show no modification from the normal. As molting progresses the microvilli become so large and so complex that they may aptly be called cell processes.

I have not examined molting in the nymphs. Contrary to Osche's (1963) suggestion that adults do not molt, I have observed molting adults in Dr. J. Teague Self's collection, and specimens in a number of early states of molting were obtained during this study.

Ultrastructural aspects of molting, as described in Section C above, are arthropodan in nature. Reviews on arthropodan molting are provided by Wigglesworth (1948a), Richards (1951), Locke (1964), and Cottrell (1964).

As in the insects (Locke, 1964), the number of ribosomes in the epidermal cells of P. crotal increases greatly during molting. The complex molting fluid is secreted by the general epidermal cells (Richards, 1951).

It appears in my micrographs that dark material is being dissolved from the endocuticle by the molting fluid (Figs. 13 and 22 through 26). Its removal does not change the appearance of the cuticle. It is doubtful that this is

the equivalent of the epidermally secreted electron dense material described in insects (Noble-Nesbitt, 1968).

Some dissolution of endocuticle is reported in all arthropods (Richards, 1951). The dissolved material is resorbed by the subcuticular cells and is used nutritionally. The amount of cuticle resorbed varies from a "few" per cent of the total cuticle dry weight up to 90% (Richards, 1951). Ecdysis is thus both an absorptive and secretory process. Much resorption would be unnecessary nutritionally in pentastomes.

The formation of an ecdysal membrane as described by Passoneau and Williams (1953) was not observed by me, but it may occur in later molting stages than I had available.

Although muscle insertions show variations between arthropod groups, the insertions in P. crotali are arthropodan. Tonofibrils pass directly from the sarcoplasm into the endocuticle without penetrating any intervening epidermal cells.

Myofilaments are continuous with the tonofibrils only rarely. The two are not separated by sarcolemma (Figs. 5 and 15) but interdigitate as broad papillae (Fig. 14).

The sarcolemma of arthropod muscle cells is generally believed to be continuous with the basement membrane and the muscle cells do not penetrate the epidermal layer (Woods, 1929; Richards, 1951). Thus, tonofibrils in arthropods either squeeze between or pass through the epidermal cells.

Haffner (1924a) described a similar situation in P. armillatus.

In P. crotali the coelomic boundary layer covers the sarcolemma and the muscle cells themselves become a part of the subcuticular layer of cells (Fig. 5). As described for Arthropods by Hinton (1948), the muscles in P. crotali appear to attach (or remain attached) at molting, retaining the structural insertion in the old cuticle until the new one is formed (Fig. 22).

Apophyses, as observed in Kiricephalus by Richards (1951), are not present in P. crotali. The exocuticular formation in Fig. 4 may be indicative of their former presence, but muscle insertions are not related to these formations.

The bulb-shaped structure seen in Figs. 30 and 31 appears to be neuroepithelial although my micrographs do not contain sufficient evidence to prove this. I suggest that it may be a proprio-mechanoreceptor. It does not appear to be the equivalent of any of the sensilla pictured from pentastomes at the light level (Haffner, 1926; Heymons, 1935). Further, it does not appear to correspond to any specific receptor pictured from other invertebrates (Bullock and Horridge, 1965). Wide varieties in sensilla morphology between pentastome species have been noted by workers in our laboratory.

This bulb-shaped structure superficially resembles cristae ampullares of the mammalian ear (Wersäll, 1956). The

cup-shaped cell would be neural, corresponding to the neural calyx. The dense cellular element would be the equivalent of the hair cell, its displacement providing the stimulus indicative of pressure on the cuticle or, perhaps, strain between the cuticle and the subcuticular epidermal layer. I can offer no explanation of the appearance of the internum of the dense element.

I further suggest that the so-called "stigmata," long thought to be glandular, may also be neuroepithelial sensory structures. In spite of certain evidence for osmoregulatory and secretory functions, neither has been proved (Self, 1969).

Doucet (1965) reported that the collar stains identically to his exocuticle. I have noted the same at the light level after staining with both Hematoxylin and Eosin as well as Giemsa. I believe that this is indicative of the homogeneity observed between the collar and the intermediate cuticular zone in the present study. Noting the continuity of the central disc with the exo- and epicuticles, this gives the collar and the intermediate cuticular zone a continuous spatial relationship with the exocuticle. Thus, cuticular form is modified, but not broken. Glandular secretions have no opening through which they could leave the structure!

The emptying of the "gland" under adverse conditions of tonicity (Doucet, 1965) could be indicative of only the weakness of the cuticle at this point, which would be

expected since the entire endocuticle is lacking. Under adverse conditions the plug-like central disc could be pushed out of its location within the collar. I suspect that this has occurred in the many cases where light microscopical examinations have pictured the collar as being a hollow tube. My retention of the central disc may be a result of the rapidity with which ultrastructural fixatives act upon the tissues.

The general structure of the "stigmata" resembles a number of neuroepithelial structures, perhaps most notably the taste bud (Engström and Rytzner, 1956) and the organ of Corti (Engström and Wersäll, 1953). It also however, resembles the hypodermal glandular cell of the bacillary band in Trichiurid nematodes (Wright, 1963) except that there, the central disc is lacking.

Speculating on the mode of stimulus reception of these structures is as enigmatic as is their true function. On the basis of the emptying phenomenon (Doucet, 1965) it seems that the central disc is somewhat free to move inward and outward against the collar (perhaps like a piston and cylinder wall). This movement could result from internal/external tonicity differentials and, through pressure changes on the infradiscal material, transmit the pressure information to the cell processes of the two modified epidermal cells.

These cells resemble neuroepithelial cells, and this has lead to my interpretation but it has not been proved by this study.

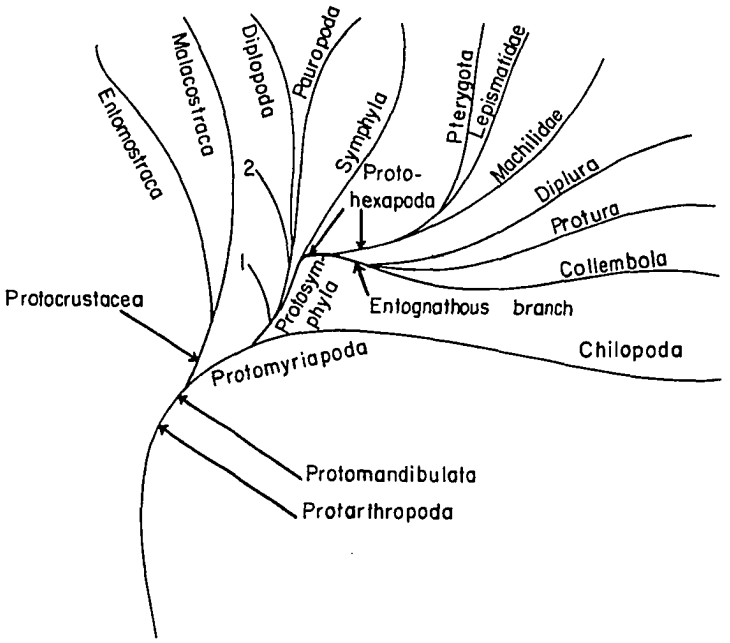
I propose that, as Doucet (1965) suggests, the so-called "stigmata" of the pentastomes do function in osmoregulation. Their role may be in sensing osmotic pressure differentials and stimulating other effector organs however, rather than being the effector organs themselves as has so long been suspected.

Any attempt at using ultrastructural observations in the elucidation of the phylogenetic origin of the pentastomes must be tempered by the knowledge that parasites often show adaptations to their specialized environments that have no relationship to the structural characteristics of their free-living ancestors.

Osche's (1963) eloquent analysis of the question placed the group next to the Pauropoda and Diplopoda (also possibly the Symphyla).

Tracing body wall characteristics alone, I placed the Pentastomida closer to the Onycophora than to the Tardigrada, their cuticle being more simplified than either. Further, I located their origin near those of the Pauropoda and the Collembola (cf. Tiegs 1947 and Noble-Nesbitt, 1963).

Indicating such a phylogenetic origin on the same "tree" used by Osche (1963) places my conclusion (arrow #1) in close agreement with his (arrow #2) (Text Fig. 1).



After Osche (1963), taken from Snodgrass (1938).

In light of the completeness of Osche's phylogenetic analysis the fact that I arrived at a point in such close agreement with his is probably due only to the significance of the cuticular system in the life functions of all arthropods and their relatives, the pentastomes.

CHAPTER V

SUMMARY

The first application of electron microscopy to body wall components of a pentastomid endoparasite confirms the relationship between the phyla Pentastomida and Arthropoda, and elucidates cuticular functions in this helminth.

Standard ultrastructural techniques allow the resolution of three layers in the cuticles of adults and infective nymphs: epicuticle, exocuticle and endocuticle. The epicuticle is some 300 A thick in adults and appears to be bipartite. In nymphs it is a 400 A homogeneous layer.

The fibrillar exocuticle of adults is 2 to 8 microns thick and indistinctly divided into superficial and deep zones. In nymphs it is a homogeneous 0.5 micron layer, modified into spines. The 8 to 30 microns endocuticle in adults appears lamellate due to parabolic chitin fibril orientation, and is separated from the exocuticle by a dense 600 A intermediate cuticular zone. In nymphs the 5 to 9 microns endocuticle contains chitin fibrils in wave-like formations and is only separated from the exocuticle at irregular intervals.

Pore canals, apophyses and apodemes are absent from all stages.

Subcuticular cells in adults are attenuated, highly interdigitated, and present irregular surfaces to the coelomic cavity. Nuclei are rarely seen in adult epidermal cells, indicating large cells or perhaps syncitia. Muscle cells are striated, extend between the subcuticular epidermal cells to the endocuticle, and insert into the endocuticle by means of an intracellular tonofibrillar attachment process. Mitochondria, granular ER and free ribosomes abound in both cell types while Golgi complexes appear to be absent.

Specialized cells in the subcuticular layer appear to be sensory. Intercellular junctions include specialized desmosomes and septate fasciae adherentes. No cuticular glands nor oenocytes are seen.

No basement membrane is present under the subcuticular cell layer in adults. A 500 to 600 A layer of amorphic homogeneous material coats all cell surfaces adjacent to the coelom including those of muscle cells and the reproductive tracts.

Molting in adults is begun by elaboration of microvilli into more structured cell processes. Subcuticular cells become very electron-dense and the plasma membranes facing the endocuticle become widened. A subcuticular space develops and enlarges to over two microns in width prior to new cuticle formation. Dense material appears to be

dissolved out of the endocuticle and released into the subcuticular space.

Subcuticular cells in nymphs are columnar but appear to extend into pleomorphic basal portions. Epidermal cells are uninucleate and contain mitochondria, smooth ER and, rarely, Golgi complexes. Desmosomes are the only attachment specializations observed. Sections of nymph muscle cells were not obtained, but two types of sensory specializations are described. Coelomic spaces are rudimentary and were not observed. No basement membrane is present under the apical portion of the epidermal cells.

A bulb-shaped receptor resembling cristae ampullares is described in the nymph as a possible proprio-/mechanoreceptor.

"Stigmata," long thought to be glandular, are described from nymphs and adults as possible osmotic pressure sensors.

INDEX DESCRIPTORS: Porocephalus crotali; Pentastomida; ultrastructure; Arthropoda; cuticle; body wall; muscle insertions; sensilla; lack of basement membrane; intercellular junctions.

LITERATURE CITED

Andersen, S. O. & Weis-Fogh, T. (1964). Resilin. A rubber-like protein in arthropod cuticle. pp. 1-65 in Beament, J. W. L. & Wigglesworth, V. B. Advances in Insect Physiology. Vol. 2. New York: Academic Press.

Baccetti, B. & Rosati, F. (1971). Electron microscopy on tardigrades. III. The integument. Journal of Ultrastructure Research 34, 214-243.

Bauer, H. (1933). Mikroskopisch-chemischer Nachweis von Glykogen und einigen anderen Polysacchariden. Zeitschrift für mikroskopisch-anatomische forschung 33, 143-160.

Blower, G. (1950). Aromatic tanning in the myriapod cuticle. Nature 165, 569.

Bouligand, Y. (1965). Sur une architecture torsadée répandue dans de nombreuses cuticles d'arthropodes. Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences. Paris 261, 3665-3668 +4 plates.

Bullock, T. H. & Horridge, G. A. (1965). Structure and Function in the Nervous Systems of Invertebrates. Vols. I & II. 1719 pp. San Francisco: Freeman & Co.

Chandler, A. C. & Read, C. P. (1961). Introduction to Parasitology with Special References to the Parasites of Man. 10th ed. 822 pp. New York: Wiley & Sons.

Chitwood, B. G. & Chitwood, M. B. (1950). An introduction to Nematology. 2d ed., Section I, Anatomy. 213 pp. Baltimore: Monumental Printing Co.

Coggeshall, R. E. (1966). A fine structural analysis of the epidermis of the earthworm Lumbricus terrestris L. Journal of Cell Biology 28, 95-108.

Cottrell, C. B. (1964). Insect ecdysis with particular emphasis on cuticular hardening and darkening. pp. 175-218 in Beament, J. W. L., Treherne, J. E. & Wigglesworth, V. B. Advances in Insect Physiology Vol. 2. New York: Academic Press.

Crompton, D. W. T. & Lee, D. L. (1965). The fine structure of the body wall of Polymorphus minutes (Goeze, 1782) (Acanthocephala). Parasitology 55, 357-364 +4 pates.

Crowe, J. H., Newell, I. M. & Thompson, W. W. (1970). Echiniscus viridis (Tardigrada): fine structure of the cuticle. Transactions of the American Microscopical Society 89, 316-325.

Dennell, R. (1960). Integument and Exockeleton. Ch. 14, pp. 449-472 in Waterman, T. H. The Physiology of Crustacea. Vol. I. New York: Academic Press.

Doucet, J. (1965). Contribution a l'étude anatomique, histologique et histochimique des pentastomes (Pentastomida). Mémoires Office de la Recherche Scientifique et Technique Outre-Mer, Paris 14, 1-150.

Drach, P. (1953). Structure des lamelles cuticulaires chez les crustaces. Compte Rendu Hebdomadaire des Séances de l'Académie des Sciences. Paris 237, 1772-1774.

Engström, H. & Rytzner, C. (1956). The fine structure of taste buds and taste fibres. Annals of Otology, Rhinology and Laryngology 65, 361-375.

Engström, H. & Wersäll, J. (1953). Structure of the organ of Corti. I. Outer hair cells. Acta Oto-laryngologica 43, 1-10.

Esslinger, J. H. (1962). Development of Porocephalus crotali (Humboldt, 1808) (Pentastomida) in experimental intermediate hosts. The Journal of Parasitology 48, 452-456.

Haffner, K. von. (1924a). Beitrage zur Kenntnis der Linguatuliden. IV. Die Körpermuskulatur von Porocephalus armillatus. Zoologischer Anzeiger 59, 270-276.

Haffner, K. von. (1924b). Beiträge zur Kenntnis der Linguatuliden. V. Die Drüsen von Porocephalus armillatus. Zoologischer Anzeiger 60, 126-136.

Haffner, K. von. (1926). Die Sinnesorgane der Linguatuliden, nebst einer Betrachtung über die systematische Stellung dieser Tiergruppe. Zeitschrift für Wissenschaftliche Zoologie 128, 201-252 +1 plate.

Hett, M. L. (1924). On the family Linguatulidae. Proceedings of the Zoological Society of London 1924, 107-159.

Heymons, R. (1935). Pentastomida. pp. 1-268 in Bronn, H. G. Klassen und Ordnungen des Tierreichs. Vol. 5, Sec. 4, Book 1. Leipzig: Akademische Verlagsgesellschaft M. B. H.

Hinton, H. E. (1948). On the origin and function of the pupal stage. Transactions of the Royal Entomological Society of London 99, 395-409.

Laverack, M. S. (1963). The Physiology of Earthworms. International Series of Monographs on Pure and Applied Biology. Division: Zoology, Kerhut, G. A. (editor). Vol. 15, 206 pp. New York: Macmillan Co.

Lee, D. L. (1966). The structure and composition of the helminth cuticle. pp. 187-254 in Dawes, B. Advances in Parasitology. Vol. 4. New York: Academic Press.

Lillie, R. D. (1954). Histopathologic Technic and Practical Histochemistry. 2nd edition, 501 pp. New York: Blakiston Co.

Lillie, R. D. & Greco, J. (1947). Malt diastase and ptyalin in place of saliva in the identification of glycogen. Stain Technology 22, 67-70.

Lison, L. (1960). Histochimie et cytochimie animale. Principes et méthodes. Vols. I & II, 842 pp. Paris: Gauthier-Villars.

Lison, L. & Vokaer, R. (1949). Sur la détection histochimique du glycogène des cellules vaginales chez la femme. Annales d'Endocrinologie 10, 66-72.

Locke, M. (1961). Pore canals and related structures in insect cuticle. Journal of Biophysical and Biochemical Cytology 10, 589-618.

Locke, M. (1964). The structure and formation of the integument in insects. pp. 379-470 in Rockstein, M. The Physiology of Insecta. Vol. III. New York: Academic Press.

Millard A. & Rudall, K. M. (1960). Light and electron microscope studies of fibres. Journal of the Royal Microscopical Society 79, 227-231 +1 plate.

Neville, A. C. (1967). Chitin orientation in cuticle and its control. pp. 213-286 in Beament, J. W. L., Treherne, J. E. & Wigglesworth, V. B. Advances in Insect Physiology. Vol. 4. New York: Academic Press.

Nicoli, R. M. (1963). Phylogénèse et Systématique le phylum des Pentastomida. Annales de Parastiologie Humaine et Comparee 38, 483-516.

Noble-Nesbitt, J. (1963). The fully formed intermoult cuticle and associated structures of Podura aquatica (Collembola). Quarterly Journal of Microscopical Science 104, 253-270 +4 plates.

Noble-Nesbitt, J. (1968). Aspects of the structure, formation and function of some insect cuticles. pp. 3-16 in Beament, J. W. L. & Treherne, J. E. Insects and Physiology. New York: American Elsevier Publishing.

Osche, G. (1963). Die systematische Stellung und Phylogenie der Pentastomida. Embryologische und fergleichend-anatomische Studien an Reighardia sternae. Zeitschrift für Morphologie und Ökologie der Tiere 52, 487-596.

Passonneau, J. V. & Williams, C. M. (1953). The moulting fluid of the cecropia silkworm. Journal of Experimental Biology 30, 545-560.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17, 208-212.

Richards, A. G. (1951). The Integument of Arthropods. The Chemical Components and Their Properties, the Anatomy and Development, and the Permeability. 411 pp. Minneapolis: University of Minnesota Press.

Richards, A. G. (1953). Structure and function of the integment. pp. 1-22 in Roeder, K. D. Insect Physiology. New York: Wiley and Sons.

Robson, E. A. (1964). The cuticle of Peripatopsis moseleyi. Quarterly Journal of Microscopical Science 105, 281-299.

Rudall, K. M. (1955). The distribution of collagen and chitin. Symposia of the Society for Experimental Biology 9, 49-71 +3 plates.

Runham, N. W. (1961). Investigations into the histochemistry of chitin. Journal of Histochemistry and Cytochemistry 9, 87-92.

Runham, N. W. (1962). Further investigations on the histochemistry of molluscan chitin. Journal of Histochemistry and Cytochemistry 10, 504.

Salthouse, T. N. (1962). Histochemistry and staining of chitin. Journal of Histochemistry and Cytochemistry 10, 109.

Schulze, P. (1932). Linguatulida, Zungenwürmer. in Brohmer, P. Fauna von Deutschland. Ein Bestimmungsbuch unserer Heimischen Tierwelt. 4th ed. 459 pp. Leipzig: Quelle & Meyer.

Self, J. T. (1969). Biological Relationships of the Pentastomida; a bibliography on the Pentastomida. Experimental Parasitology 24, 63-119.

Slifer, E. H. & Sekhon, S. S. (1963). The fine structure of the membranes which cover the egg of the grasshopper, Melanoplus differentialis, with special reference to the hydropyle. Quarterly Journal of Microscopical Science 104, 321-334 +8 plates.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26, 31-43.

Tiegs, O. W. (1947). The development and affinities of the Pauropoda, based on a study of Pauropus silvaticus. Quarterly Journal of Microscopical Science 88, 165-267 +9 plates (Part I) and 275-336 +2 plates (Part II).

Wersäll, J. (1956). Studies on the structure and innervation of the sensory epithelium of the cristae ampullares in the guinea pig. Acta Oto-Laryngologica, Supp. 126, 1-85.

Wigglesworth, V. B. (1934). The physiology of the cuticle and of ecdysis in Rhodnius prolixus (Triatomidae, Hemiptera); with special reference to the function of the oenocytes and of the dermal glands. Quarterly Journal of Microscopical Science 76, 269-318.

Wigglesworth, V. B. (1948a). The structure and deposition of the cuticle in the adult mealworm, Tenebrio molitor L. (Coleoptera). Quarterly Journal of Microscopical Science 89, 197-217 +2 plates.

Wigglesworth, V. B. (1948b). The insect cuticle. Biological Reviews 23, 408-451.

Wigglesworth, V. B. (1957). The physiology of insect cuticle. Annual Review of Entomology 2, 37-54.

Wigglesworth, V. B. (1965). The Principles of Insect Physiology. 741 pp. London: Metheuen.

Wood, R. L. (1959). Intercellular attachment in the epithelium of Hydra as revealed by electron microscopy. Journal of Biophysical and Biochemical Cytology 6, 343-351 +6 plates.

Woods, W. C. (1929). The integument of the larva of the alder flea beetle. Bulletin of the Brooklyn Entomological Society 24, 116-123.

Wright, K. A. (1963). Cytology of the bacillary bands of the nematode Capillaria hepatica (Bancroft, 1893). Journal of Morphology 112, 233-259.