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ORGANIZED FILAMENTS IN THE ADHESIVE SYSTEM OF *Macrostomum tuba* GRAFF, 1882 (PLATYHELMINTHES, MACROSTOMIDA)

Marina Silveira[1] and Pedro Henrique Arruda Aragão[2]

[1]Laboratory of Electron Microscopy, Institute of Physics, University of São Paulo (USP), São Paulo, SP, Brazil
[2]Laboratory of Electron Microscopy and Microanalysis, Department of Physics,
State University of Londrina (UEL), Londrina, PR, Brazil.

ABSTRACT

The adhesive organs or "duo-gland adhesive organs" of platyhelminths are formed by a specialized epithelial cell and extensions of two gland cells. These organs are used for temporary fixation of the organism to surfaces in aquatic habitats. The mechanisms involved in adhesion to and release from a given surface depend on secretions produced by the glands; less is known about the involvement of cytoplasmic filaments in the anchoring cell itself. In this study, we examined the structure of the adhesive organs present in the tail plate of *Macrostomum tuba* Graff, 1882 (Platyhelminthes, Macrostomida), a freshwater, free-living flatworm. Scanning and transmission electron microscopy allowed elucidation of the three-dimensional organization of the adhesive system, especially of the microvilli that formed the outer collar (or papilla), which was endowed with a fibrous core. Electrical stimulation caused the flatworms to extend their papillae above the ciliated surface. The use of tannin- and diamine-containing fixatives showed that the filamentous array contained tonofilaments and actin filaments**.** Tonofilaments concentrate in the axis of each microvillus; actin filaments, about 7-8 nm thick, spread out towards the periphery. Scanning images demonstrated the finger-like shape of the papillae, about 7-8 μm high, with a terminal opening. Microvilli followed a straight course along the surface.

Key words*:* Adhesive organs, *Macrostomum tuba*, microfilaments, ultrastructure

INTRODUCTION

Small aquatic flatworms and other invertebrates possess a variety of adhesive systems [4,10,22,25] that are important for temporary attachment to substrates [27,29]. Marine macrostomid flatworms that live in interstitial habitats rely on adhesive organs and a well developed muscular system to withstand water currents [15]. The "duo-gland" adhesive organ was described in detail by Tyler [25-27] based on electron microscopy and cytochemistry. These studies consistently demonstrated the presence of filaments that were considered to be tension-bearing elements [27]. Whittington and Cribb [29] reviewed the structural and functional aspects of adhesive devices found in Platyhelminthes, including the duo-gland type. Morphological and ultrastructural data on these and other small organs are useful for systematics and for ascertaining phylogenetic relationships within the group [4-6,14,17,27].

The adhesive papillae of *Macrostomum tuba* stand out above the ciliated epithelial covering of the tail plate, as already demonstrated using scanning electron microscopy, [Aragão P.H.A., Doctoral thesis, University of São Paulo, São Paulo, Brazil]. In this report, we provide additional information to complement data previously reported for this system [19]. We also describe the use of some novel techniques, including electrical stimulation, sonic waves, fixatives containing lysine or tannin, and osmium impregnation. Other intracellular or extracellular filaments unrelated to the adhesive organ were also seen in thin sections and were useful for comparative purposes.

Correspondence to: Dr. Marina Silveira
Laboratório de Microsocopia Eletrônica, Instituto de Física, Universidade de São Paulo (USP) CP 66318, 05315-970, São Paulo, SP, Brazil. Tel: (55) (11) 3091-6854, Fax: (55) (11) 3813-4334. E-mail: msilveir@if.usp.br

Some of the data reported here were presented at the 7th International Symposium on the Biology of the Turbellaria held in Turku in 1993 [19].

MATERIAL AND METHODS

Transmission electron microscopy (TEM)

Specimens of *M. tuba* maintained in freshwater aquaria were fixed using a conventional glutaraldehyde/OsO_4 procedure, and also using the following procedures: **a)** 2.5 h fixation in 1.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.1, with post-fixation in 2% OsO_4 followed by 1% tannin in 0.05 M cacodylate-buffer, pH 7.0, for 1.5 h, and washing in 1% Na_2SO_4 [20] (referred to as fixative SS); the samples were then dehydrated and embedded in Polybed or LX-112 epoxy resin, **b)** fixation in 2% glutaraldehyde in 0.06 M cacodylate-buffer, pH 7.4, containing 0.1 M lysine (Sigma, freshly diluted in 0.06M cacodylate-buffer, pH 7.4), for 1 h. After several washes in cold acetate/veronal buffer, pH 7.6, 0.275 M, the specimens were fixed overnight in 2.5% glutaraldehyde in 0.06 M cacodylate buffer, pH 7.4, washed again in buffer, post-fixed in 2% OsO_4, washed in cold water, stained in 2% aqueous uranyl acetate, dehydrated and embedded in resin [1,2].

Thin-sections were stained with uranyl acetate and lead citrate and examined with a Philips CM-200 electron microscope operated at 160 or 200 kV, or a Siemens Elmiskop 101 electron microscope operated at 100 kV. Original negatives of selected images were used for measurements at magnifications of up to X 50,000 with a Peak glass reticle ruled at 0.01 mm and mounted on a 15 X magnifier.

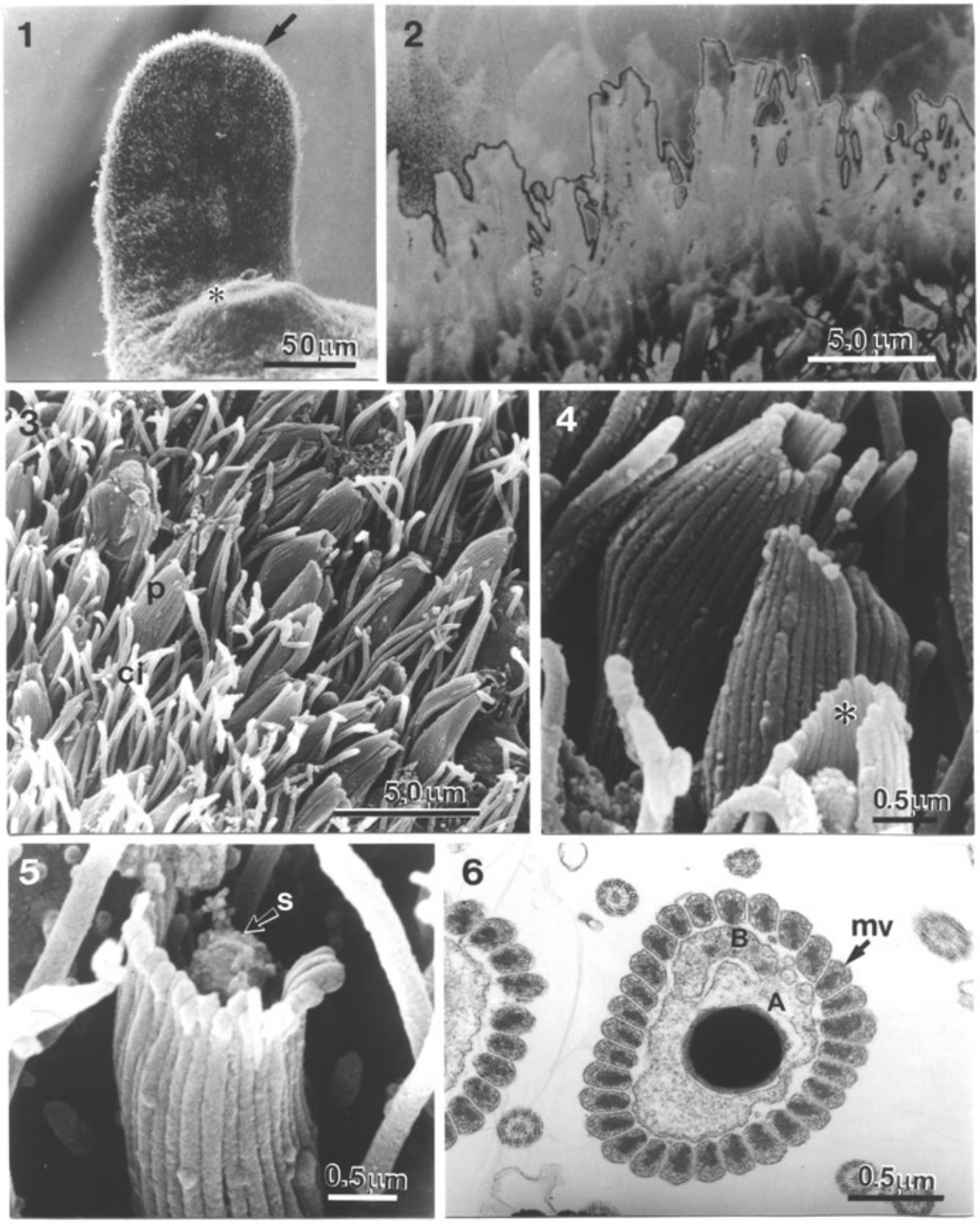
Scanning electron microscopy (SEM)

The flatworms were immersed for 15 min at 4°C in the "instant fixative" of Tamm and Tamm [23] that consisted of 0.5 mL of 25% glutaraldehyde, 2 mL of 0.045 M cacodylate buffer, pH 7.2, 8 mg of NaCl and 2.5 mL of aqueous 4% OsO_4 (referred to as fixative TT). After fixation, the flatworms were washed in sodium cacodylate buffer containing 0.35 M NaCl, and post-fixed in 2.5% glutaraldehyde in the same buffer. Conductivity was improved by using the osmium/thiocarbohydrazide/osmium (OTO) impregnation method [11,12]. The specimens were dehydrated in ethanol, critical point-dried using CO_2 and then mounted with silver paste, sputtered with gold, and studied in a Jeol JSM-840A scanning electron microscope operated at 25 kV. This procedure was applied to flatworms swimming in a Petri dish, or to flatworms subjected to a 220 V (d.c.) potential, applied for ~1-5 s; this voltage corresponded to a current of ~1.6 to 7.4 mA passing through the flatworms. To remove the epidermal cilia, some flatworms fixed in fixative TT and impregnated with metal as described above were dehydrated in 100% ethanol and sonicated in a small, closed cuvette in a model T586 Sonicor tank, at 220 V and 50 Hz for 1-10 s, followed by critical point-drying [Aragão P.H.A., Doctoral thesis].

RESULTS

The adhesive system of *M. tuba* consisted of a number of papillae located on the ventral surface of the tail plate (Fig. 1). These organs were masked by the dense ciliary covering in quiescent or free-swimming individuals, but became visible when the flatworms were disturbed. The papillae corresponded to the distal end of a specialized epithelial cell, the anchor cell, that surrounded the projections of two types of gland cells to form the duo-gland adhesive system. Figures 2 and 3 show scanning electron micrographs of these elements. Extended papillae of *M. tuba* were ~7-8 μm high, almost cylindrical in shape, and varied in diameter, depending on the level at which they were sectioned. Each papilla was formed by closely apposed microvilli that were particularly evident in the electron micrographs (Figs. 3 and 4). Cross-sections showed up to 25-29 microvilli arranged in a regular fashion around two projections of gland cells, one viscid and another releasing gland. In specimens of *M. tuba* fixed under electrical stimulation, the papillae appeared stretched although the microvilli were not dissociated (Fig. 3); only a few of the papillae were ruptured longitudinally (Fig. 4, asterisk). Figure 5 shows the release of secretion (**s**) from a papilla opened by sonication for 5 s (10 W waves). In one experiment (not shown), the papillae were skewed but well distended.

Figures 1. Face view of the caudal plate of a specimen of *Macrostomum tuba* folded in an upright position relative to the body. T&T fixative (see text). Note the bright, outermost rim of papillae (**arrow**); (*****) - gonopore. SEM; **2.** Close view of another specimen in which a single scanning line enhanced the profile of a row of papillae. Glutaraldehyde/OsO_4 fixative. SEM with "contour level" processing of the video image; **3.** The adhesive border of a caudal plate showing the papillae (**p**) stretched out among the cilia (**ci**). The animal was stimulated with an electrical current and then fixed with T&T. SEM; **4.** Higher magnification of the central area shown in Figure 3. The wall of each papilla is formed by adjoining, parallel microvilli, one of which has split-off (**asterisk**). SEM; **5.** Detail of another papilla releasing a secretion granule (**s**) through its opening. The microvilli are slightly swollen at their tips. The sample was sonicated for 5 s and then fixed with T&T. SEM; **6.** Transverse sections of papillae containing processes of the viscid gland (**A**) and release gland (**B**). Each peripheral microvillus (**mv**) contains a dense core. Boyles' fixative. TEM.


1
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Thin sectioned microvilli showed a similar structural organization that was bound by delicate connections of extracellular material. This outer coat, which was well preserved by tannin- and lysine-containing fixatives (Figs. 6, 7 and 9), was probably responsible for maintaining the integrity of the papillae, as shown by TEM and SEM. Each microvillus had adense core containing fine, longitudinally oriented filaments. In cross-section, these filaments had a characteristic distribution that consisted of *central* and *peripheral* elements. The peripheral elements had a mean diameter of 75 Å and were generally displaced towards the outermost surface of the papilla (Fig. 9, arrow), whereas the central elements formed a very dense packing of less distinct units (Fig. 9, asterisk). The peripheral filaments were better identified after glutaraldehyde/ lysine fixation, which indicated that they were actin filaments.

The second category of rather compact filaments corresponded to tension-bearing elements and were assumed to be tonofilaments. In longitudinal sections, this central bundle was not well-resolved (Fig. 7) and there was only the suggestion of a wavy alignment (of filaments) within the bundle, with the bundle itself being oriented parallel to the microvillar axis. Towards the base of the anchoring cell, some "frayed" filaments apparently came in contact with septate junctions of the epithelial cell (Fig. 8).

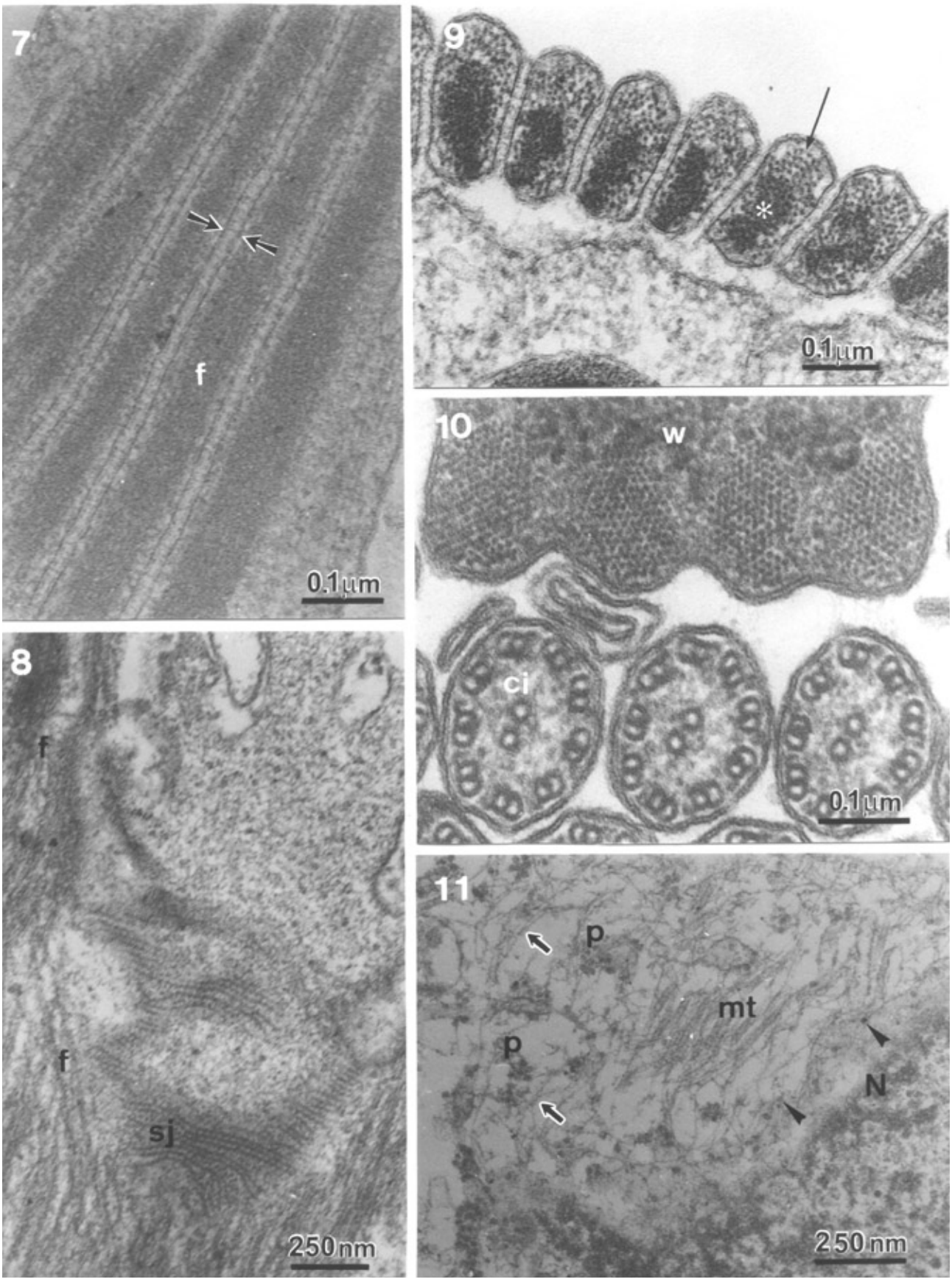
Other filament systems were also clearly seen in sections of *M. tuba*. The protonephridium contained a prominent population of longitudinally oriented filaments that filled the inner and outer ribs of the flame bulbs and reinforced the walls of the nephridial ducts. In tannin fixed samples, the filaments (ca. 90 Å in diameter) were organized into regular, hexagonal bundles (Fig. 10). In cross-section, these bundles approximated the dimensions of ciliary axonemes and provided a robust support for the cytoplasm. Cytoskeletal elements (microtubules and microfilaments of different "parenchymal cells") were particularly well preserved following fixation in glutaraldehyde/lysine. Figure 11 shows a network of filaments less than 100 Å thick that was retained against the depleted cytosol. Some of these filaments were seen adhering to nuclear and cell membranes, while others were in contact with ribosomes and polysomes. The filaments were rather sinuous and ran close to bundles of microtubules. The identity of these cells remains uncertain because they were extensively lysed during fixation.

DISCUSSION

The adhesive system represented by a specialized epithelial cell (the anchor cell) and two associated gland cells is an interesting arrangement encountered in several plathelminths and other lower metazoans [4,10,22,29]. This system includes a collar of microvilli with axial filaments that very likely correspond to the tension-bearing elements described by Tyler [27]. In this work, we examined the distal end of the anchor cell of *M. tuba,* a barrel shaped protrusion of the specialized epithelial cell that has an insunk cell body. This anchor cell was not studied in great detail here, but the general organization of its papilla approximated that of *Macrostomum* sp. II (see Tyler [26]).

In *M. tuba*, the adhesive organs occurred almost exclusively in the flattened tail plate, which is a strategic location since this region is richly supplied with muscles and nerve endings [15,16]. In the macrostomid *Macrostomum hystricinum marinum* studied by Rieger *et al.* [16], the dorso-ventral muscles in this region are forked and help to flatten the tail plate, thereby reinforcing adherence of the papillae to the substrate. Interestingly, the adhesive plate of *Macrostomum* spp. is the only part of these flatworms that can completely regenerate within two weeks of surgical removal [18]. In *M. tuba*, the

Figures 7. Longitudinal section of a papilla near its distal portion. Note the regular interspacing of the microvilli bound together through thin periodic bridges (between **arrows**). The plasma membrane and the axial dense core of filaments (**f**) are apparent. Boyle's fixative. TEM; **8.** Basal portion of a papilla within an "anchor" cell. Note the filament bundles (**f**) extending deep beyond the free portion of the papilla, with some of them in contact with a septate junction (**sj**). Boyle's fixative. TEM; **9.** Detail of a row of microvilli in cross-section. Note the two distinct zones within each microvillus consisting of a distal grouping of rather disperse filaments ~70 in diameter (**arrow**), and an inner, denser region of less distinct units (✱). A delicate extracellular coat binds the microvilli together. Boyle's fixative. TEM; **10.** Section through a nephridial canal of *M. tuba*. Fixation was done according to Simionescu and Simionescu [20] and the section was oxidized in H_2O_2 before staining. Note the regular packing of (90 Å diameter) filaments within undulations of the canal wall (**w**). **ci** - cilia. TEM; **11.** Cytoskeleton of a parenchymal cell after extraction. The cytoskeleton consisted of microtubules (**mt**), branched filaments (ca. 100 Å in diameter) in contact with the tangentially sectioned nuclear membrane (**N**, **arrowheads**), and a plasma membrane (not shown). Polysomes (**p**) were trapped in the filamentous net. Boyle's fixative. TEM.


7
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9
*
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w
ci
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adhesive system reacts immediately to mechanical and electrical stimulation. Theoretically, since this species lives in calm environments, its adhesive system might be functionally less developed that those of flatworms living in turbulent waters. However, this was found not to be the case. Fixation with glutaraldehyde/tannin [20] resulted in good preservation of the extracellular coat, thereby ensuring firm coherence of the microvilli around the collar.

Based on a cytochemical/functional study of various adhesive organs, Tyler [27] stated that the secretion of the viscid gland contained proteinaceous and glycan components. This secretion was used to fix the papilla to the substrate, whereas release from the substrate was provided by an as yet uncharacterized secretion from the second gland. However, the true functions of both secretions remain speculative [29].

The distribution of filaments within the microvilli and their variable appearance in transverse and longitudinal sections indicated the presence of actin and intermediate filaments. The diameter of the former filaments (75 Å in our preparations) was very close to the 80 Å typical of actin. These filaments were well preserved by the tannin-containing fixative SS, as also reported by LaFountain *et al.* [9] for other systems. F-actin bundles are involved in several biological functions, including changes in cell shape and motility [3,7,21,24]. Using fluorescent-labeled phalloidin and confocal microscopy, Pfistermüller and Tyler [13] demonstrated the presence of F-actin in specialized collared sensory receptors of the acoel *Convoluta pulchra,* in which the actin filaments most likely occurred within the microvilli; this distribution was reminiscent of that seen here for *M. tuba*. Microvilli in the stereocilia of several turbellarians also contain reinforced axial fibrils [**6**]. The compact filamentous bundle seen in *M. tuba* and interpreted here as tonofilaments was very similar to that of cochlear hair cells [24]. The two types of filaments (actin and tonofilaments) would contribute to the extensibility and tensile strength of the microvilli.

The addition of tannic acid or lysine to glutaraldehyde preserves actin in a variety of non-muscle cells [1,2,9,21,24], although the functions of actin and associated proteins in non-muscle cells remain to be clearly defined [7]. The two procedures that we used here were reliable and useful for actin identification, but do not supersede immuno-fluorescence techniques [3] or the heavy meromyosin "decoration method" of Ishikawa *et al*. [8]. According to Boyles and co-workers [1,2], diamine stabilizes actin against damage during post-fixation with osmium. Other filaments detected in our preparations of *M. tuba* included cytoplasmic microtubules (diameter: 250 Å) and thin, flexible filaments of the cytoskeletal network. In the nephridial system, a prominent array of filaments 90-100 Å diameter was seen and most likely corresponded to the fine striations mentioned by Watson *et al.* [28] for this same species and considered to be supporting structures.

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