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ADDITIONAL ULTRASTRUCTURAL OBSERVATIONS OF THE GILL EPITHELIUM OF THE WATER FLEA *DAPHNIA MAGNA* WITH REFERENCE TO IONIC AND MACROMOLECULAR TRANSPORT

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ABSTRACT. The ultrastructural features of the gill epithelium of adult *Daphnia magna* are consistent with their dual function of ion transport from the surrounding medium to the hemolymph and transport of macromolecules from the hemolymph towards the cuticle. The thin cuticle of the gill epithelium displays short, thin epicuticular tubercles and pits. Silver grains penetrate the cuticle in $AgNO_3$ treated specimens. The single layer of flat epithelial cells is of one type only, the dark cells. The epithelial cells display extensive infoldings of the apical and basal plasma membranes associated with mitochondria and delicate, folded lateral membranes enclose narrow intercellular spaces. They have large irregular nuclei with prominent nucleoli. Their cytoplasm is rich in mitochondria, rough and smooth endoplasmic reticulum, microtubules, and vesicles, while Golgi complexes are sparse. The basal cytoplasm displays dense tubular elements, coated vesicles, multivesicular bodies, and lysosomes. These ultrastructural features are characteristic of ion transporting epithelia and of cells engaged in protein and lipid synthesis.

Keywords: *Daphnia magna*, gill epithelium, membrane infoldings, mitochondria, rough endoplasmic reticulum, dense tubules, multivesicular bodies, coated vesicles, cuticle, lysosomes

INTRODUCTION

Animals living in fresh water are faced with the problem of maintaining osmotic pressure and ionic composition of body fluids higher than that of the surrounding medium. To solve this problem aquatic animals have developed mechanisms to absorb ions into their blood (Krogh 1939; Rankin & Davenport 1981). Aquatic larvae of dipterous genera, such as *Aedes* and *Chironomus*, have developed anal papillae that absorb sodium and chloride ions into the hemolymph (Stobbart 1960; Wright 1975). In many crustaceans the gills play a role not only in respiration but also act as osmoregulatory organs (Prosser 1973). In hyperosmotic crustaceans sodium influxes from dilute media have been reported (Sutcliffe 1968; Harris 1970). The activity of Na^+/K^+ ATPase enzyme, which is strongly implicated in sodium transport, has been measured in the pleopod gills of isopod *Idotea wosnesenskii*, acclimated to different media, suggesting their role in inward ionic transport from dilute media (Holliday 1988). Kirschner in his review article (2004), Freire et al. (2008), Bianchini & Wood (2008), and Tsai & Lin (2014) all have implicated gills and other structures in the ion regulatory mechanisms of crustaceans.

Ultrastructural studies of ion transporting organs include larval anal organ/papillae of semiaquatic *Drosophila melanogaster* and aquatic *Chironomus tentans* (Jarial 1987, 1995); chloride cells of *Callibaetis* sp. (Ephemeroptera) (Komnick & Able 1971); labium of *Cenocorixa bifida* (Jarial 2003); "gills" (= endopodites) of terrestrial isopods like *Oniscus* (Kűmmel 1981); and the gills of crayfish, *Pacifastacus leniusculus* (Morse et al. 1970), of euryhaline Chinese crab, *Eriocheir sinensis* (Barra et al. 1983), and of shrimp, *Penaeus japonicus* (Bouaricha et al. 1994). In these studies epithelial cells of these organs were characterized by highly folded apical and basolateral plasma membranes associated with many mitochondria. The extensive plasma membrane infoldings associated with mitochondria provide increased surface area and energy for active ion transport. Similar ultrastructural features are exhibited by transporting epithelia throughout the animal kingdom (Berridge & Oschman 1972).

The ultrastructure of the gill epithelium of *Daphnia magna* acclimated to different salinities was investigated by Kikuchi (1983). The purpose of the present study was to further elucidate the

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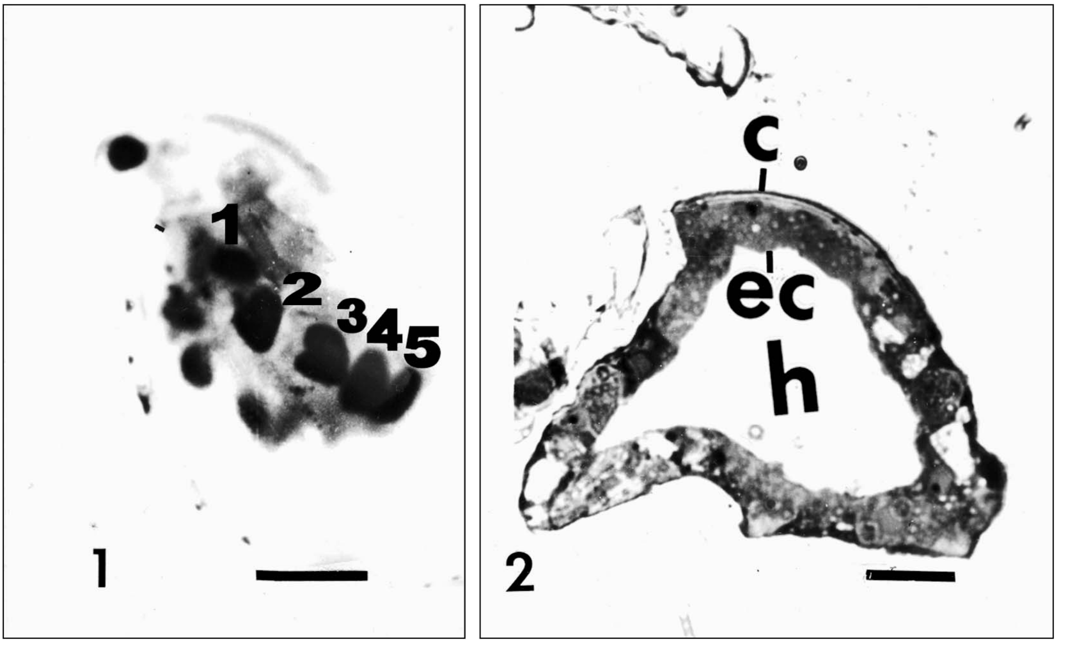


Figures 1 & 2.—Gills of *Daphnia magna*. 1. Lateral view of a specimen treated with $AgNO_3$ solution showing five darkly stained gills (1-5). Scale bar = 0.3 mm. 2. Light micrograph of a cross section (2 μm) of a gill stained with azure II. c = cuticle; ec = epithelial cells; h = hemocoel. Scale bar = 20 μm.

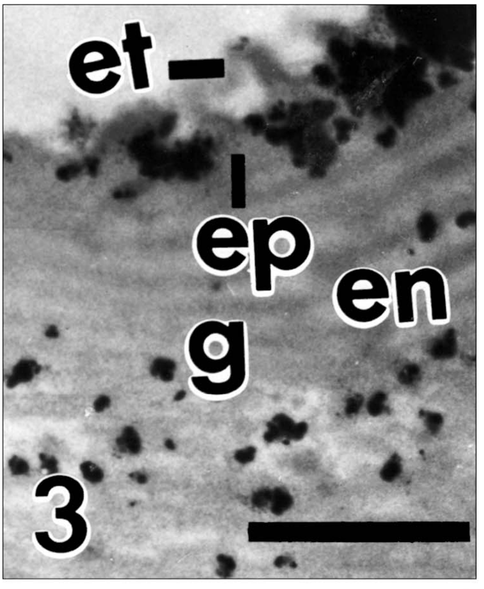
subcellular features of the gill epithelium of *Daphnia magna* and to relate them with their function in ionic uptake from the external medium and transport of macromolecules from the hemolymph to the cuticle.

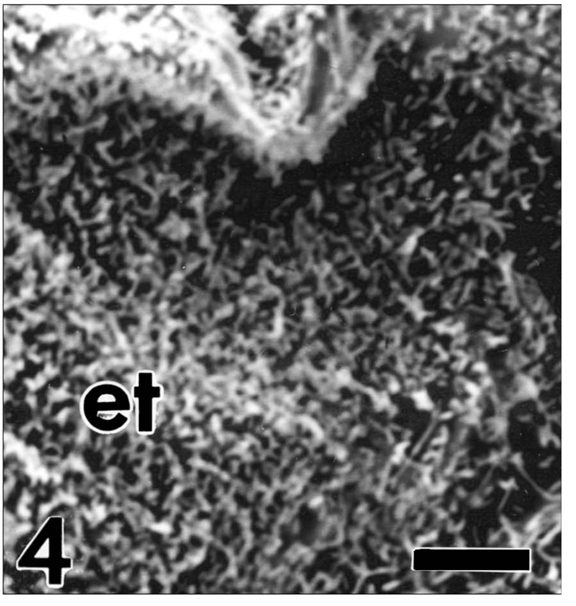
METHODS

Adult *Daphnia magna* used in this study were obtained from Carolina Biological Supply Company, Burlington, NC. Six specimens were used immediately upon arrival. For transmission electron microscopy (TEM) the animals were fixed by immersion at room temperature in 2.5% glutaraldehyde and 2% paraformaldehyde (1:1) in 0.1M cacodylate buffer at pH 7.4 (Millonig 1976), washed in two changes of buffer, post-fixed in 1% osmium tetroxide in the same buffer, and washed in two changes of buffer. Permeability of silver ions was demonstrated by the silver nitrate ($AgNO_3$) method of Ewer & Hattingh (1952). A few animals were quickly rinsed in distilled water and immersed in 1% $AgNO_3$ solution for 5 minutes followed by a quick rinse in distilled water to remove any adherent $AgNO_3$. The animals were then exposed to Kodak D-19 developer to reduce silver to metallic black silver, rinsed in distilled water, photographed, and immersed in the aforementioned fixative mixture. The gills were removed with sharp scissors, dehydrated in an ethanol series, transferred to propylene oxide, embedded in LX112 (Ladd Industries) (Luft 1961), and polymerized at 60°C overnight. Ultrathin sections were cut on a Porter-Blum MT-2 ultra-microtome, stained with uranyl acetate and lead citrate, and examined with a Hitachi 600 TEM. For scanning electron microscopy (SEM), similarly fixed material was chemically dried in hexamethyl-disilazane (Polysciences), coated with gold/palladium, and examined with a Cambridge S-90 SEM. Thick (2 μm) sections were cut, stained with azure II, and examined with an American optical series 20 light microscope (LM).

RESULTS

Adult *Daphnia magna* have five pairs of small sac-like gills that protrude externally from their thoracic appendages. They become darkly stained when exposed to dilute silver nitrate solution (Fig. 1). Each gill measures approximately 110 μm in its long axis. The gill epithelium is composed of a single layer of large, flattened





Figures 3 & 4.—Features of the gill cuticle. 3. Electron micrograph of the gill cuticle from a $AgNO_3$ treated specimen composed of two layers, an inner laminated endocuticle (en) and outer epicuticle (ep) bearing short, narrow epicuticular tubercles (et) facing externally. Silver grains (g) penetrate both layers of the cuticle. Scale bar = 1 μm. 4. Scanning electron micrograph of the external surface of a gill displaying small epicuticular tubercles (et). Scale bar = 10 μm.

epithelial cells containing dense cytoplasm. Lateral membranes of these cells are not distinguishable by light microscopy. The epithelial cells basally are in contact with the hemocoel while the cells apices are covered by a thin cuticle measuring about 1.5 μm in thickness (Fig. 2). In electron micrographs, the cuticle is composed of two layers, an inner lamellate endocuticle and an outer epicuticle (Fig. 3). In transmission and scanning micrographs, the epicuticle displays short, narrow epicuticular tubercles and pits (Figs. 3, 4, 5 inset, 9). In specimens exposed to silver nitrate solution, the silver grains are seen penetrating both layers of the cuticle to reach underlying epithelium (Fig. 3). The apical plasma membrane of the epithelial cells located under the thin cuticle is organized into numerous narrow, closely spaced infoldings, their tips closely associated with many large mitochondria (Fig 5). The cytoplasm contains numerous mitochondria, smooth and rough endoplasmic reticulum, vesicles, microtubules, ribosomes, and dense tubules (Figs. 5, 7, 9). The epithelial cells contain centrally placed large, irregularly shaped nuclei with prominent nucleoli (Fig. 6). The basal plasma membrane (facing the hemocoel) is supported by a conspicuous basal lamina that is elaborately infolded to form invaginations closely associated with mitochondria. These basal membrane infoldings form a labyrinth of intracellular channels that anastomose freely in the basal cytoplasm (Figs. 8, 9). The basal cytoplasm contains free ribosomes, abundant rough endoplasmic reticulum in the form of whorls and cisternae, multi-vesicular bodies, dense tubules, coated and smooth vesicles, and lysosomes, while the Golgi complexes appear sparse (Figs. 7, 9). The delicate lateral membranes are folded forming interdigitations among adjacent epithelial cells. They enclose narrow intercellular spaces and are joined apparently by septate desmosomes that link the lateral membranes (Figs. 5, 7, 8). Figure 9 summarizes the ultrastructural organization of the gill epithelium of *D. magna* as revealed by this study.

DISCUSSION

The silver staining technique has been used to locate ion transporting organs in a number of aquatic insects and crustaceans (Krogh 1939; Ewer & Hattingh 1952; Copeland 1967; Jarial et al. 1969; Morse et al. 1970; Barra et al.

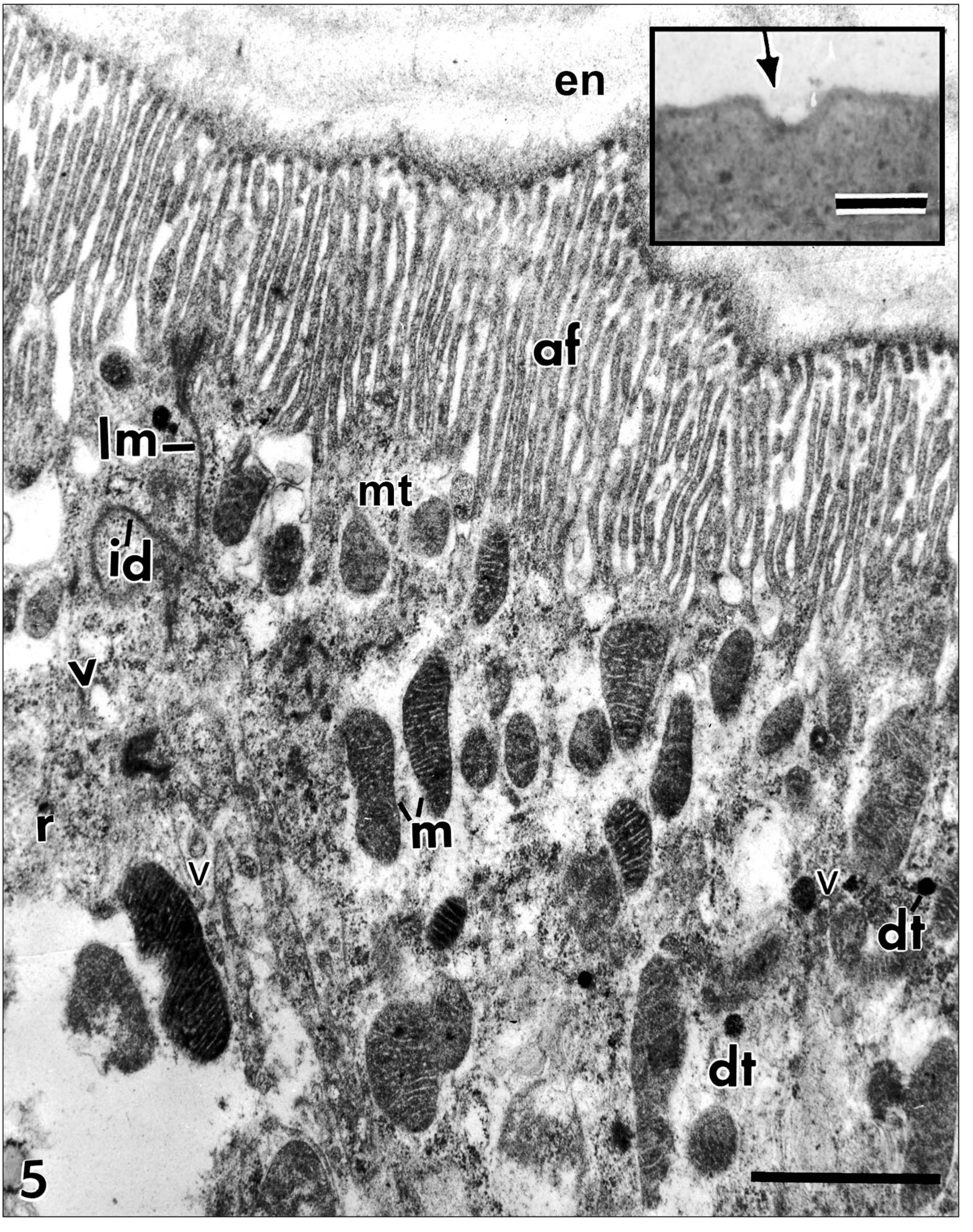
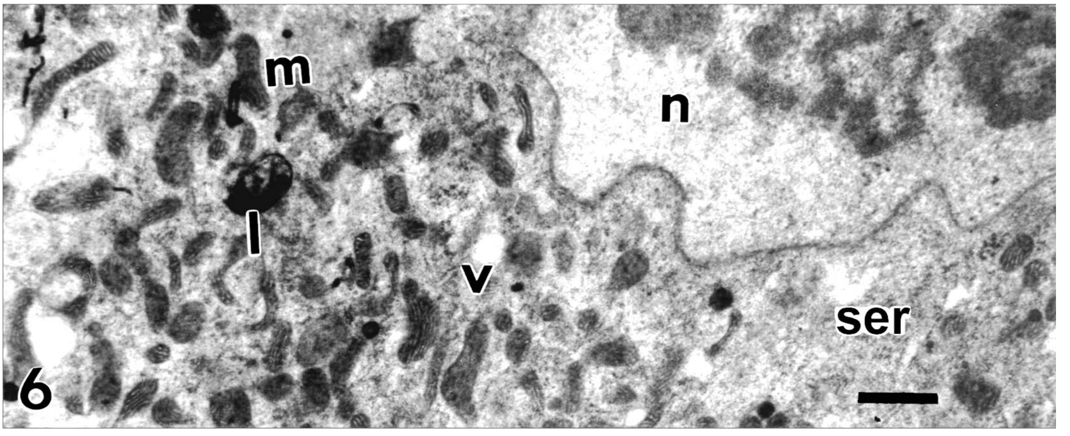
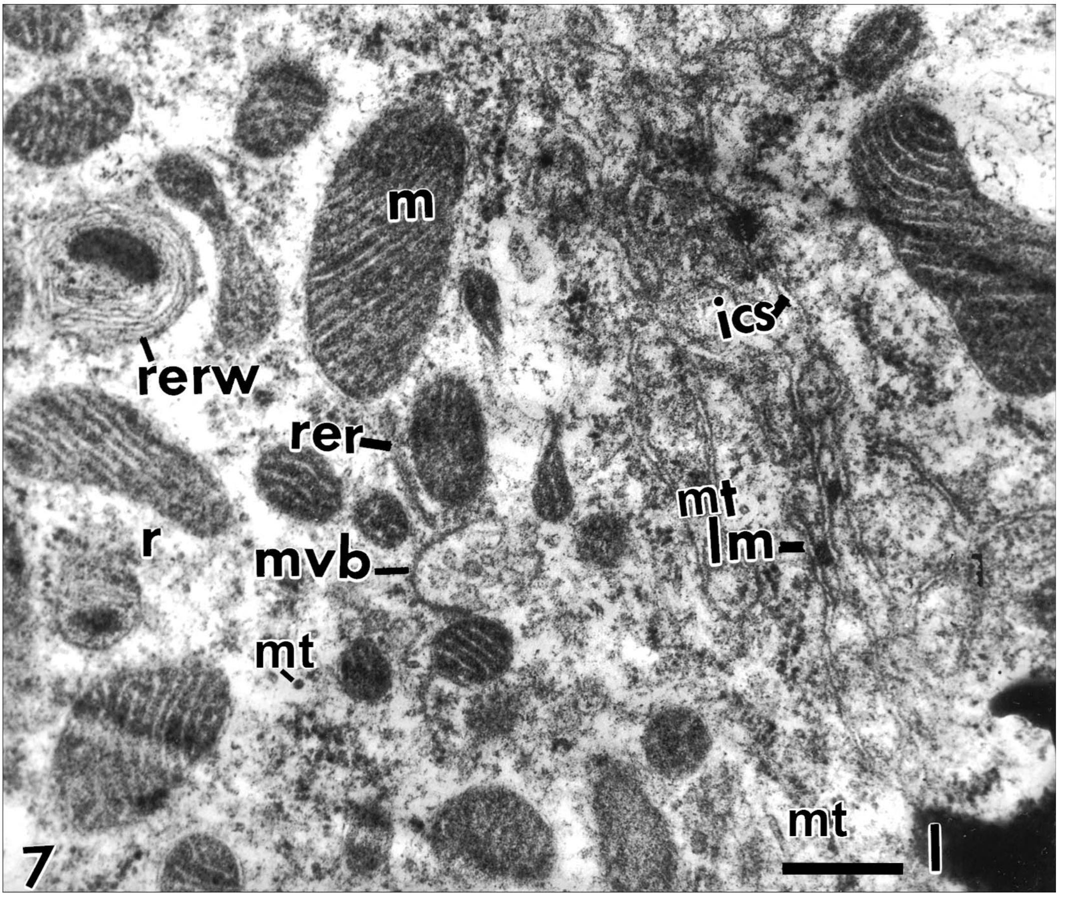


Figure 5.—Electron micrograph of apical region of a gill epithelial cell showing numerous apical membrane infoldings (af) and large mitochondria (m). en = endocuticle; dt = dense tubules; id = interdigitation between adjacent cells; lm = lateral membranes; r = ribosomes; mt = microtubules; v = vesicle. Scale bar = 0.5 μm. Inset: A portion of epicuticle showing a pit (arrow). Scale bar = 0.2 μm.





Figures 6 & 7.—Cytoplasmic detail of the central region of gill epithelial cells. 6. Nuclear region of an epithelial cell showing a large, irregular nucleus (n), numerous mitochondria (m), smooth endoplasmic reticulum (ser), lysosomes (l), and vesicles (v) in the cytoplasm. Scale bar = 1 μm. 7. Higher magnification electron micrograph shows large mitochondria (m), rough endoplasmic reticulum whorls (rerw), and cisterns (rer) in the cytoplasm. ics = intercellular space; l = lysosome; lm = lateral membrane; mt = microtubules; mvb = multivesicular body; r = ribosomes. Scale bar = 0.25 μm.

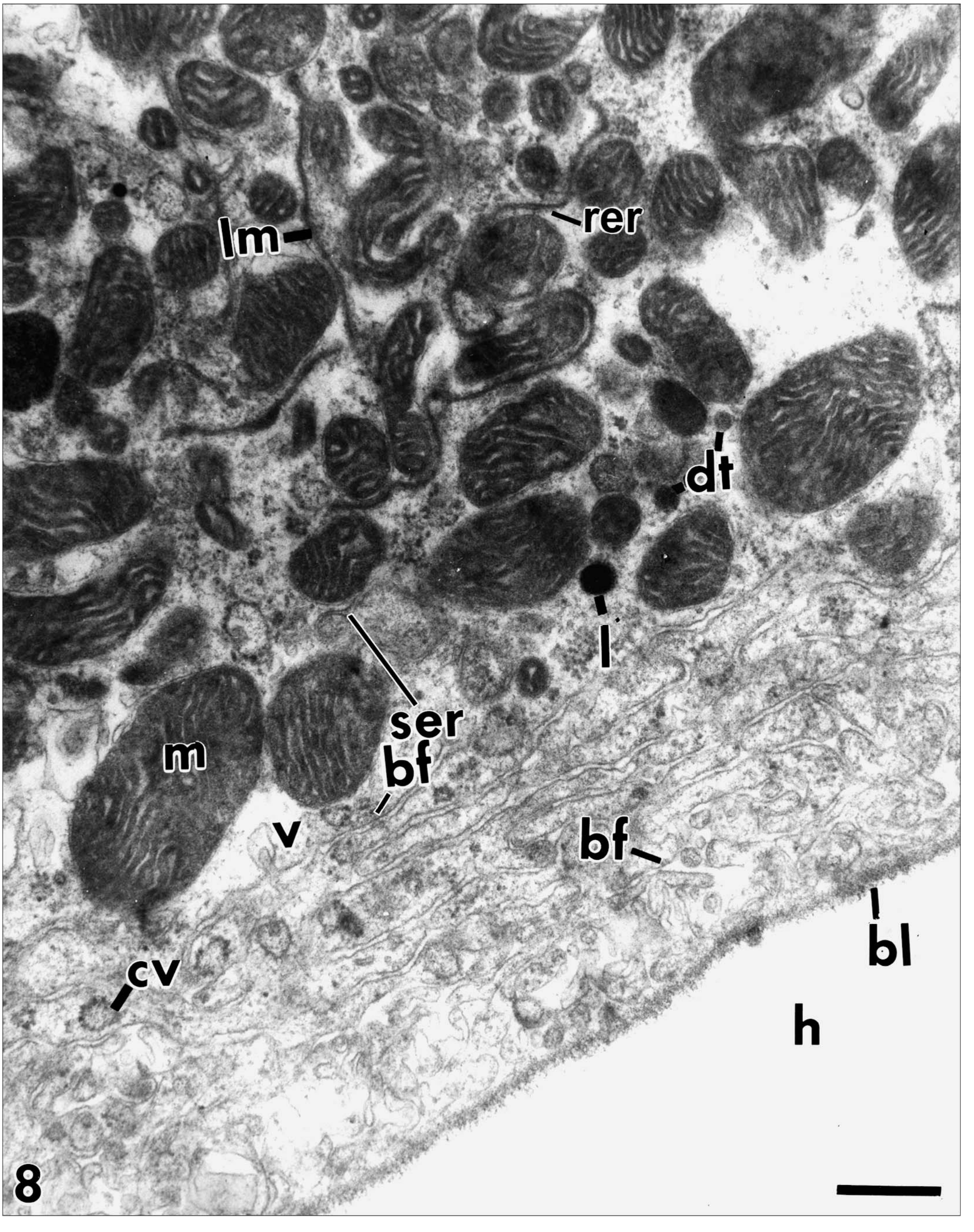


Figure 8.—Electron micrograph of the basal region of a gill epithelial cell displaying basal membrane infoldings (bf) resting on a prominent basal lamina (bl). cv = coated vesicle; dt = dense tubule; h = hemocoel; l = lysosome; lm = lateral membrane; m = mitochondrion; ser = smooth endoplasmic reticulum; v = smooth vesicle. Scale bar = 0.25 μm.

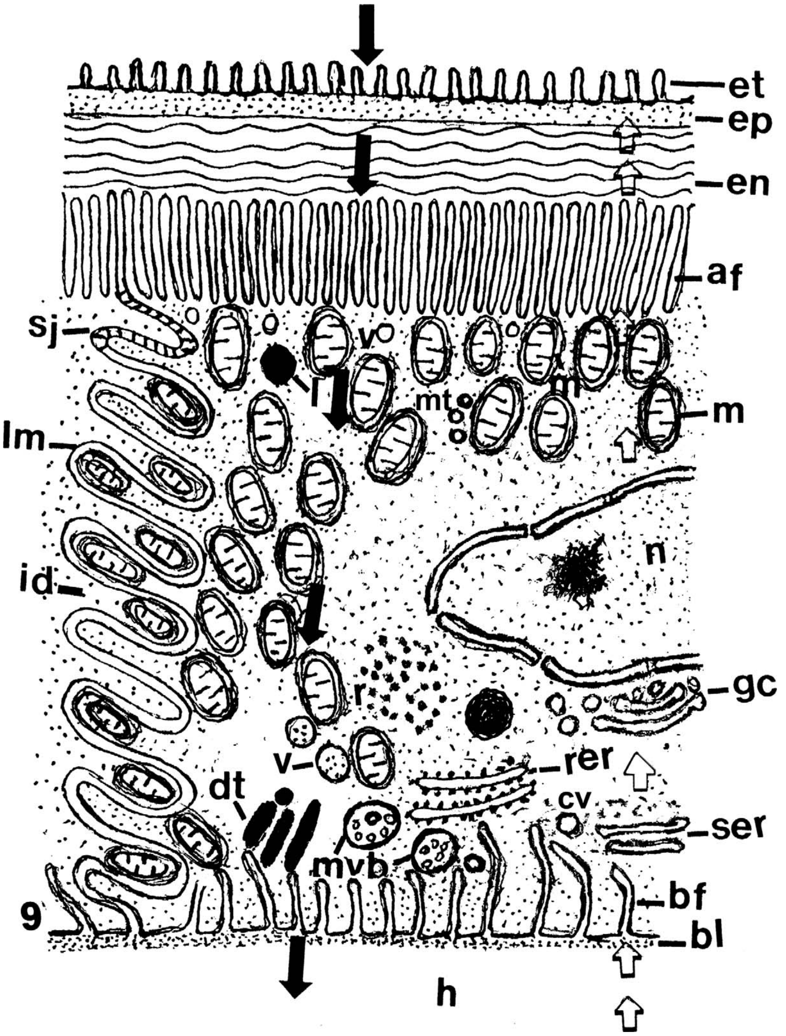


Figure 9.—Diagrammatic representation of the ultrastructural features of a gill epithelial cell of *Daphnia magna*. Solid arrows represent transport of ions from external medium into the epithelial cells and open arrows represent transport of macromolecules from the hemocoel into the epithelial cells and the cuticle. af = apical membrane infoldings; bf = basal membrane infoldings; bl = basal lamina; cv = coated vesicles; dt = dense tubules; en = endocuticle; ep = epicuticle; et = epicuticular tubercles; gc = golgi complex; h = hemocoel; id interdigitations; l = lysosome; lm = lateral membrane; m = mitochondria; mt = microtubules; mvb = multivesicular bodies; n = nucleus; r = ribosomes; rer = rough endoplasmic reticulum; ser = smooth endoplasmic reticulum; sj = septate junction; v = vesicles.

1983; Kikuchi 1983; Holliday 1998). X-ray microanalysis has demonstrated that in tissues stained with $AgNO_3$, chloride ions released from the tissues were captured as AgCl precipitates on the tissue surfaces (Barra et al. 1983). Silver staining of the gills and ultrastructural localization of silver grains in the epicuticle, endocuticle, and apical region of the epithelial cells suggest that the gill epithelium of *D. magna* is permeable to chloride ions and possibly to other ions.

The ultrastructural features of the gill epithelium of *D. magna* are essentially similar to those of the dark cells of *Daphnia* described by Kikuchi (1983), but the light cells were not observed. Differences noted by us include the absence of large cytoplasmic tubules or any connection of such structures with the basal or lateral membranes. This may be due to the different fixative used in our study. Also, in the current study the intercellular spaces bounded by lateral cell membranes appear too narrow to play any significant role in fluid transport. Lastly, the basal membrane infoldings of the epithelial cells are relatively short.

Ultrastructural features of the gill epithelium of *D. magna* include extensive infoldings of the apical and basal plasma membranes, as well as folded lateral membranes that are closely associated with many large mitochondria. The later provide a large surface area and energy for fluid transport. Such ultrastructural features are commonly exhibited by epithelia that specialize in ion and water transport from dilute media (Berridge & Oschman 1972). In contrast, the salt-secreting gill epithelial cells of brine shrimp, *Artemia salina* (Copeland 1967), the epithelial cells of the pereopodal discoid osmoregulatory organ of esturine amphoid, *Melita setiflagella* (Kikuchi & Matsumasa 1995), and the epithelial cells of the anal papillae of the saltwater mosquito larva, *Aedes campestris* (Meredith & Phillips 1973), are characterized by shallow apical membrane infoldings that are not closely associated with mitochondria. These are further characterized by deep extensive basal membrane infoldings associated with many mitochondria that apparently play a role in removing excess salts from the hemolymph.

The ultrastructural organization of the gill epithelium of *D. magna* bears close resemblance to that of anal papillae of aquatic larval forms of insects (Copeland 1964; Jarial 1995) and the gills of crustacea (Barra et al. 1983; Bouaricha et al. 1994) that engage in the active transport of ions from dilute media (Stobbart 1960; Sutcliffe 1968).

The unique ultrastructural features of the gill epithelial cells of *D. magna* are the presence of dense tubular elements, coated vesicles, multivesicular bodies, and lysosomes in the basal cytoplasm. These features are characteristic of cells that take up colloidal materials and synthesize proteins (Miller 1960; Maunsbach 1966; Locke 1974). The network of basal membrane infoldings allows the uptake of macromolecules from the hemolymph into the epithelial cells. Once in the epithelial cells, the macromolecules are degraded and used in the synthesis of proteins in the well-developed rough endoplasmic reticulum (Palade 1975) and lipid synthesis in the smooth endoplasmic reticulum (Cormack 1987) to help fuel cellular metabolism and contribute to the protein and lipid constituents of the cuticle covering the gill epithelium (Hadley 1994; Neville 1998). The microtubules function in maintaining the shape of the gill epithelial cells and apparently play a role in moving organelles like vesicles in the cytoplasm (Mescher 2010).

In conclusion, the ultrastructure of the gill epithelium of *D. magna* suggests that this structure is engaged in the active transport of ions from the medium into the hemolymph to maintain osmotic constancy of the body fluids, as well as trans-epithelial transport of hemolymph macromolecules for synthesis of protein and lipid components of the cuticle covering these organs. This study provides the basis for future functional studies that may help elucidate further how the gill epithelium works.

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LITERATURE CITED

Barra, J.A., A. Pequeux & H. Willy. 1983. A morphological study on gills of a crab acclimated to fresh water. Tissue & Cell 15:583–596.

Berridge, M.J. & J.L. Oschman. 1972. Transporting Epithelia. Academic Press, New York, New York. 91 pp.

Bianchini A. & C.M. Wood. 2008. Sodium uptake in different life stages of crustaceans: the water flea *Daphnia magna Strauss*. Journal of Experimental Biology 211:539-547.

Bouaricha, N., M. Charmantier-Daures, P. Thuet, J. P. Trilles & G. Charmantier. 1994. Ontogeny of osmoregulatory structures in the shrimp, *Penaeus japonicus* (Crustacea, Decapoda). Biological Bulletin 186:29–40.

Copeland, E. 1964. A mitochondrial pump in the cells of the anal papillae of the mosquito larva. Journal of Cell Biology 23:253–263.

Copeland, E. 1967. A study of salt secreting cells in the brine shrimp (*Artemia salina*). Protoplasma 63:363–384.

Cormack, D.H. 1987. Ham's Histology, 9th ed. Lippincott, Williams & Wilkins, Philadelphia, Pennsylvania. 732 pp.

Ewer, D.W. & I. Hattingh. 1952. Absorption of silver on the gills of a freshwater crab. Nature, London 169:460.

Freire, C.A., H. Onken & J.C. McNamara. 2008. A structural functional analysis of ion transport in crustacean gills and excretory organs. Comparative Biochemistry and Physiology: A Molecular Integrative Physiology. 151:272-304.

Hadley, N.F. 1994. Water Relations of Terrestrial Arthropods. Academic Press, New York, New York. 356 pp.

Harris, R.R. 1970. Sodium uptake in the isopod *Sphaeroma rugicduda* Leach, during acclimatization to high and low salinities. Comparative Biochemistry and Physiology 32:263–273.

Holliday, C.W. 1988. Branchial Na^+/K^+-ATPase and osmoregulation in the isopod *Idotea wosnesenskii*. Journal of Experimental Biology 136:259–272.

Holliday, C.W. 1998. A protocol for silver staining ion transport epithelia of whole animals. Microscopy Today 98:12.

Jarial, M.S. 1987. Ultrastructure of the anal organ of *Drosophila* larva with reference to ion transport. Tissue & Cell 19:559–575.

Jarial, M.S. 1995. Fine structure of anal papillae in larval chironomids, *Chironomus tentans* (Diptera) with reference to ionic and macromolecular transport. Invertebrate Biology 114:324–333.

Jarial, M.S. 2003. Design of labial cuticle in *Cenocorixa bifida* Hung. (Hemiptera, Corixidae) with reference to ionic transport. Zoological Science 20:125–131.

Jarial, M.S., G.G.E. Scudder & S. Teraguch. 1969. Observation on the labium of *Corixidae* (Hemiptera). Canadian Journal of Zoology 47:713–715.

Kikuchi, S. 1983. The fine structure of the gill epithelium of a fresh-water flea, *Daphnia magna* (Crustacea, Phyllopodia) and changes associated with acclimation to various salinities. Cell Tissue Research 229:253–268.

Kikuchi, S. & M. Matsumasa. 1995. Pereopodal disc: a new type of extrabranchial ion-transporting organ in an esturine amphipod, *Melita setiflagella* (Crustacea). Tissue & Cell 27:635–643.

Kirschner, L. 2004. Review: The mechanism of sodium chloride uptake in hyperregulating aquatic animals. The Journal of Experimental Biology 207:1439-1452.

Komnick, H. & J.H. Able. 1971. Location and fine structure of the chloride cells and their porous plates in *Callibaetis spec*. (Ephemeroptera, Bactidae). Cytobiologie 4:467–479.

Krogh, A. 1939. Osmotic Regulation in Aquatic Animals. Oxford University Press, London. 242 pp.

Kűmmel, G. 1981. Fine structural indications of an osmoregulatory function of the "gills" in terrestrial isopods (Crustacea, Oniscoidea). Cell Tissue Research 214:663–666.

Locke, M. 1974. The structure and formation of the integument in insects. Pp. 123-213. *In* The Physiology of Insecta, Vol. 6. (M. Rockstein, Ed.), Academic Press, New York, New York.

Luft, J.H. 1961. Improvements in epoxy resin embedding methods. Journal of Biophysical and Biochemical Cytology 9:409–414.

Maunsbach, A.V. 1966. Absorption of ferritin by rat kidney proximal tubules: electron microscopic observations of the initial uptake phase in cells of microprofused single proximal tubules. Journal of Ultrastructural Research 16:1–12.

Meredith, J. & J.E. Phillips. 1973. Ultrastructure of the anal papillae of a saltwater mosquito *Aedes campestris*. Journal of Insect Physiology 19:1157–1172.

Mescher, A.L. 2010. Junqueiras Basic Histology, 12th ed. McGraw-Hill Company, New York, New York. 467 pp.

Miller, F. 1960. Hemoglobin absorption by the cells of the proximal convoluted tubules in mouse kidney. Journal of Biophysical and Biochemical Cytology 8:689–718.

Millonig, G. 1976. Laboratory Manual of Biological Electron Microscopy. Mario Saviolo, Vercelli, Italy. 28 pp.

Morse, H.C., P.J. Harris & E.J. Dornfeld. 1970. *Pacifastacus leniusculus*: fine structure of the arthrobranch with references to active ion uptake. Transactions of the American Microscopical Society 89:12–27.

Neville, C. 1998. Significance of insect cuticle. Pp, 151-176. *In* Microscopic Anatomy of Invertebrates. (F.W. Harrison, M. Locke & C. Neville, Eds.) Wiley-Liss, New York, New York.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. Science 189:347–358.

Prosser, C.L. 1973. Comparative Animal Physiology, 3rd ed. W.B. Saunder Company, Philadelphia, Pennsylvania. 578 pp.

Rankin, J.C. & J.A. Davenport, Eds. 1981. Animal Osmoregulation. Blackie and Sons, Ltd., Glasgow, England. 220 pp.

Stobbart, R.H. 1960. Studies on the exchange and regulation of sodium in the larva of *Aedes aegypti* (L.) II. The net transport and the fluxes associated with it. Journal of Experimental Biology 37:594–608.

Sutcliffe, D.W. 1968. Sodium regulation and adaptation to fresh water in gammarid crustaceans. Journal of Experimental Biology 48:359–380.

Tsai, J.R. & H.C. Lin. 2014. Functional anatomy and ion regulatory mechanisms of the antennal gland in a semi-terrestrial crab, *Ocypode stimpsoni*. Biology Open 3:409-417.

Wright, D.A. 1975. Sodium regulation in the larvae of *Chironomus dorsalis* (Meig.) and *Comptochironomus tentans* (Fabr.): the effect of salt depletion and some observations on temperature changes. Journal of Experimental Biology 62:121–139.

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