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Fine Structure of Salt and Water Uptake in the Land-Crab, *Gecarcinus lateralis*

D. EUGENE COPELAND

Biology Department, Tulane University, New Orleans, La., 70118, and the Marine Biological Laboratory, Woods Hole, Mass., 02543.

SYNOPSIS. The salt-absorbing tissue is found in the respiratory lamellae of the gills in the form of a highly interdigitated epithelium. The folds of the epithelium are supplied with mitochondria in the form of "mitochondrial pumps." Intercellular spaces between the folds also satisfy the morphological requirements for Diamond's model system for water transport based on his theory of standing osmotic gradients. Most of the osmoregulatory tissue is localized in the three most posterior gills, which in turn rest on the pericardial sac. It is suggested that the pericardial sac transfers ground water to the gills for salt and water absorption. The pericardial sac serves for storage of water and as a hydraulic assist during the molt.

In the last several years considerable attention has been paid to the physiology of osmoregulatory mechanisms in Crustacea (Potts and Parry, 1964; Croghan, Curra, and Lockwood, 1965; Dehnel, 1966; Gross, *et al.*, 1966; King, 1966; Quinn and Lane, 1966; Ramamurthi, 1966; Dall, 1967; Rudy, 1966, 1967; Burton, 1967; Kerley and Pritchard, 1967; Mantel, 1967; and Smith, 1967). There is agreement that the chief route of salt absorption, under hyposmotic environmental situations, is through the gills.

The histology of crab gills has been described at the level of the light microscope by Bernecker (1909), Chen (1933), Webb (1940), and Flemister (1959). Observations with the electron microscope have been made by me on the osmoregulatory tissue in the gills of the blue crab *(Callinectes sapidus),* (Copeland, 1963, 1964*b*, 1968).

Gecarcinus lateralis is unusual in being able to live indefinitely on land, obtaining its water and salts from damp sand. Bliss (1963; Bliss, *et al.*, 1966) has shown that setae on the postero-ventral surface of the crab attract water from the moist sand. The water then travels by capillary attraction in body grooves up and onto the surface of the pericardial sacs. The sacs serve as water storage organs, swelling markedly just prior to a molt. Bliss derives the very natural hypothesis that the surface of the pericardial sacs may be a site of water absorption.

This report deals with the possible roles of the gills or pericardial sacs, or both, in the uptake of water and salts, with particular emphasis on cellular fine structure.

PROCEDURES

Specimens of *Gecarcinus lateralis* were furnished through the courtesy of Dorothy Bliss. Following her experience, the crabs were kept isolated in covered plastic shoe boxes with about one inch of sand moistened with pond water. They were fed once every week with lettuce and carrots. About every other week a small piece of raw fish and half an egg shell were added. The egg shell is essential for proper hardening after molt. Crabs were adapted at least a month to the pond water to assure that the osmoregulatory mechanism of the gill would be under demand to absorb salt.

The gills proved extremely difficult to fix by normal procedures for electron microscopy. Perfusion of fixatives probably adequately fixed the internum of the gills, but the cuticle resisted penetration of the embedding plastic. Reasonable results were finally obtained by the following procedure used on crabs that had re-

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FIG. 1. Gross morphology of the gills. Anterior is to the left. Note the closer spacing of the respiratory platelets on the last two gills (VIII and IX).

cently molted (*i.e.*, within a week of the molt so that cuticle would be new).

Gills were removed and placed on absorbent cotton. Six percent glutaraldehyde (Sabatini, *et al.*, 1963) in phosphate buffer at pH 7.4 (Millonig, 1961) brought to 690 milliosmoles with sucrose (Caulfield, 1957) was dripped at room temperature on the gills for 20 minutes. The absorbent cotton rapidly pulled the fixative between the respiratory platelets, or lamellae, of the gills. The gills were then placed in vials of the same fixative at room temperature, and the vials placed on a shaking table in a refrigerator where they gradually cooled to 5°C. Fixation totaled 5 hours. Then followed six changes of phosphate buffer for a total of one hour during which time the tissue was allowed to come to room temperature. The gills were again placed on absorbent cotton, and chitinase (Worthington enzyme, 25 mg per 5 ml phosphate buffer) was permitted to drip on them intermittently. Incubation was for 20 minutes at room temperature. Incubation was terminated by immediate dripping of 2% osmic acid in phosphate buffer for several minutes, then the post-fixation was completed for one hour in a vial at room temperature. The osmotic values of all solutions, including the osmic postfix, were adjusted to 690 milliosmoles with sucrose. Embedment was in Epon 812 (Luft, 1961) and sections were stained with the uranyl acetate (Watson, 1958) and lead citrate

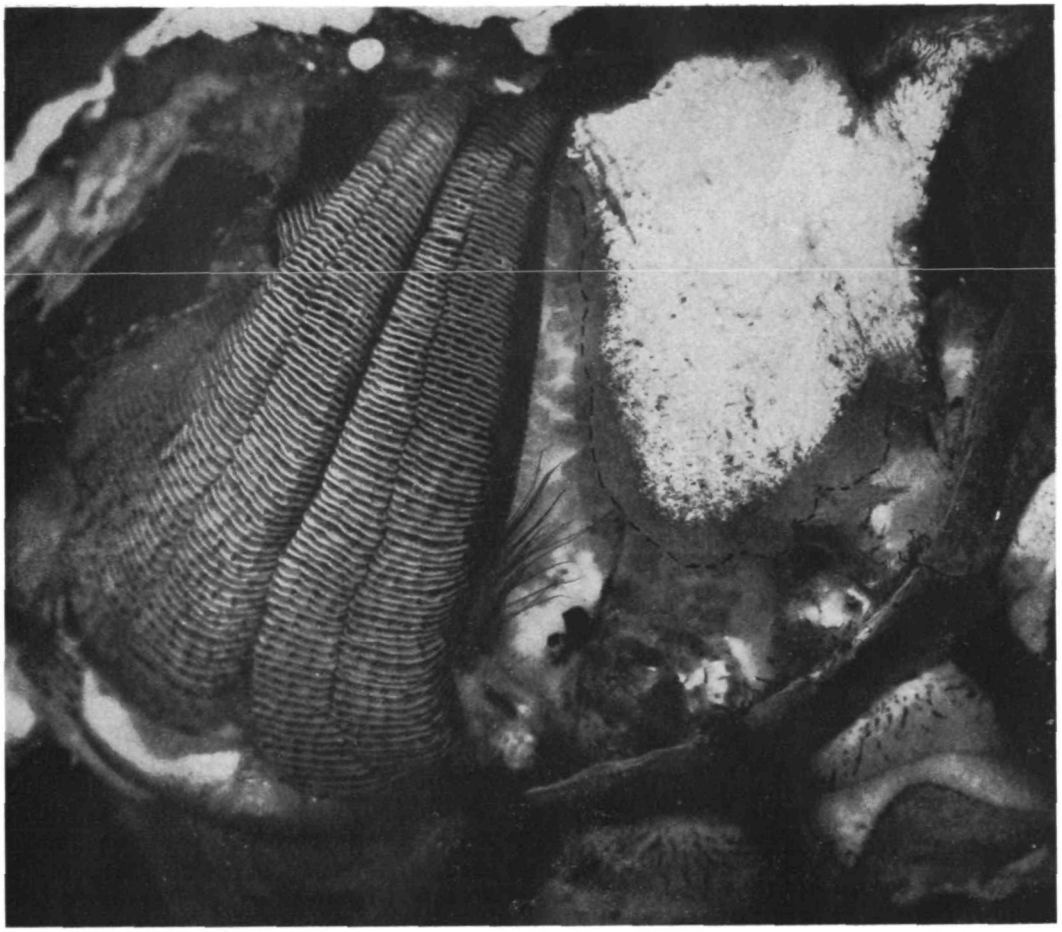

FIG. 2. Gross morphology of the pericardial sac. Gills VII, VIII, and IX are removed. The tip of gill VI is pinned back slightly to show the base of the sac. The transparent border of the sac is marked with dashes.

(Reynolds, 1963) sequence. Photos were made with the Siemens Elmiskop I.

The fixation of the pericardial sac was easily accomplished by injecting fixative with a small hypodermic needle into the reticular spaces, with essentially the same fixation times as above. Treatment with chitinase was not needed because the sac would be adequately opened by slicing it into small strips just before post-fixation.

Whole preparations for demonstrating the reticular distribution of muscle strands in the pericardial sac were made by fixing the sac overnight in the above glutaraldehyde fixative and washing it in several changes of buffer for another day. The sac was then cut into strips with a razor blade. The cut surfaces were "surface-stained" by placing the strips in a dilute aqueous solution of indulin. Photographs were made through a 32 mm Micro-tessar lens with a yellow filter to induce contrast.

Based on experience with investigations of the gill of *Callinectes* (Copeland, 1968), osmic-fixed dissections of the respiratory platelets were projected and drawn in outline. The areas of the osmoregulatory tissue and the respiratory surfaces were then determined with a Maho-Compensation-Planimeter.

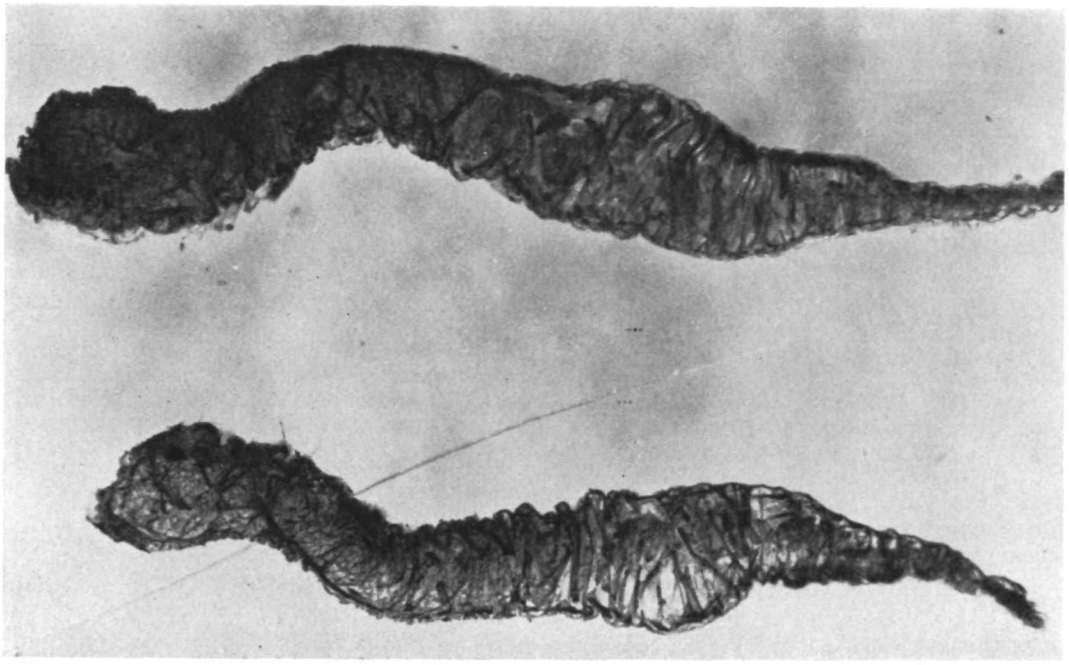

FIG. 3. Two cut surfaces of the pericardial sac, stained lightly with indulin to illustrate the proliferation of muscle strands connecting the two surfaces of the sac.

RESULTS

Gross morphology of the gills. Gecarcinus lateralis has nine pairs of gills, the first two pairs being quite small and vestigial. Figure 1 illustrates the last five pairs of gills (V to IX, inclusive). On most of the gills, the respiratory platelets, or lamellae, are kept separate by knob-like and ridge-like protrusions, thus assuring space for circulation of air. However, the respiratory lamellae on the most posterior gills (VIII and IX) are more numerous and much more closely apposed, so much so that the inter-lamellar air spaces are greatly reduced or, indeed, may be obliterated by water attracted into the interstices. The last two gills look whiter because there is less open space in which dark debris can collect.

Gross morphology of the pericardial sac. Figure 2 shows the same animal as in Figure 1, but with the three most posterior gills (VII, VIII, IX) removed. The pericardial sac, in the intermolt condition, is a flattened fold of tissue extending down from the body wall at the posterodorsal edge of the gill cavity. It tapers gradually to transparent, somewhat scalloped edges. The body of the sac is filled with a reticulum of randomly arranged muscle strands and connective tissue in which is embedded varying amounts of a white flocculent-appearing material.

Figure 3 shows two cut surfaces of the pericardial sac, stained to show the muscle strands and the connective tissue. The muscle strands run from one surface to the other in great numbers and (not illustrated) also in an irregular net just beneath each free surface of the sac.

Distribution of osmoregulatory tissue in the various gills. The tissue presumably responsible for salt absorption lines the luminal cuticular surfaces of the respiratory lamellae as a single layer of cells, with an occasional cell spanning the luminal space in the form of a pilaster cell (Fig. 4).

Though the dimensions vary, the osmoregulatory layer of cells is from ten to twenty times as thick as the rest of the endothelial layer lining the respiratory la-

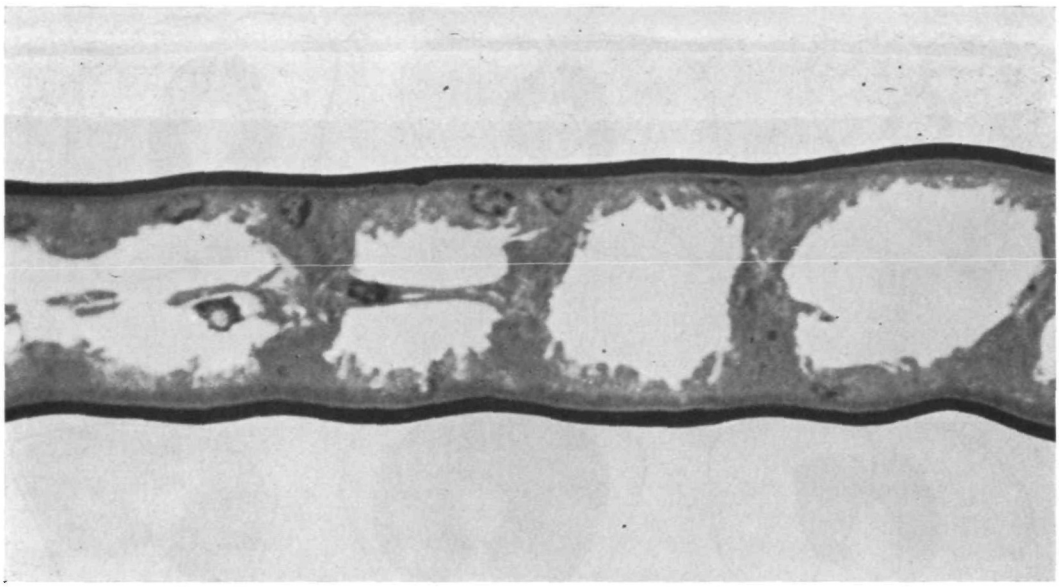

FIG. 4. Cross section of a respiratory platelet, or leaflet, in the area of the osmoregulatory tissue. The tissue forms a single cell layer lining the internum of each cuticular layer. At intervals, pilaster cells anchor the two cuticular surfaces together.

mellae. Thus, after osmic fixation, it is easy to visualize the distribution of the osmoregulatory tissue by reason of its greater density (Fig. 5).

With density as an index, the respiratory lamellae near the base of each of the major gills (the posterior seven out of nine pairs) were surveyed for relative ar-

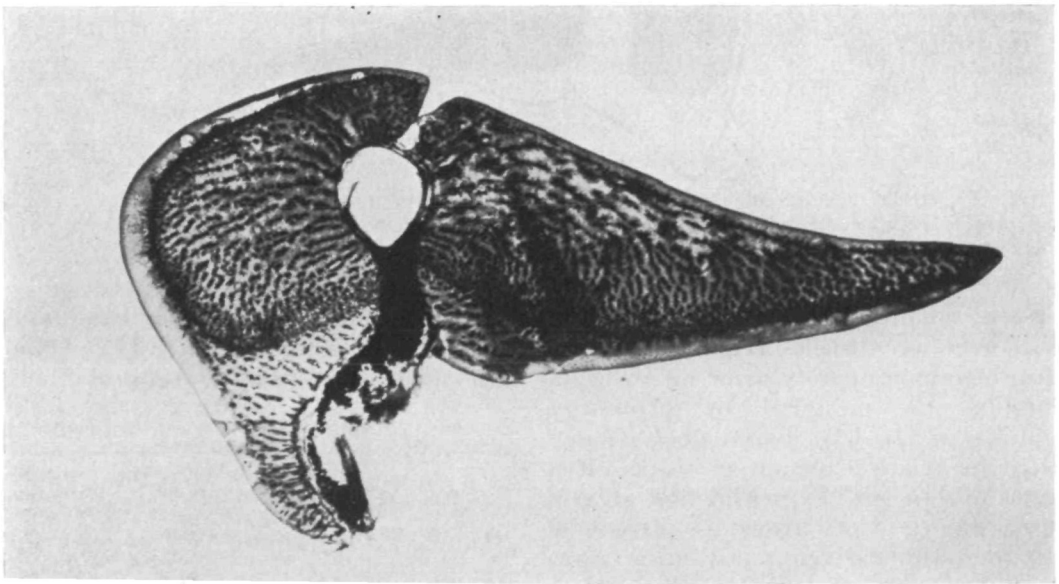

FIG. 5. Section of gill IX near its base showing a pair of respiratory leaflets, or lamellae. Afferent vessel is above and efferent is below. The more dense osmoregulatory tissue can be seen in the upper portion of the lamellae. See Figure 6 for diagrammatic depiction. The osmoregulatory patch on the right is partially obscured by surface discoloration of the cuticle.

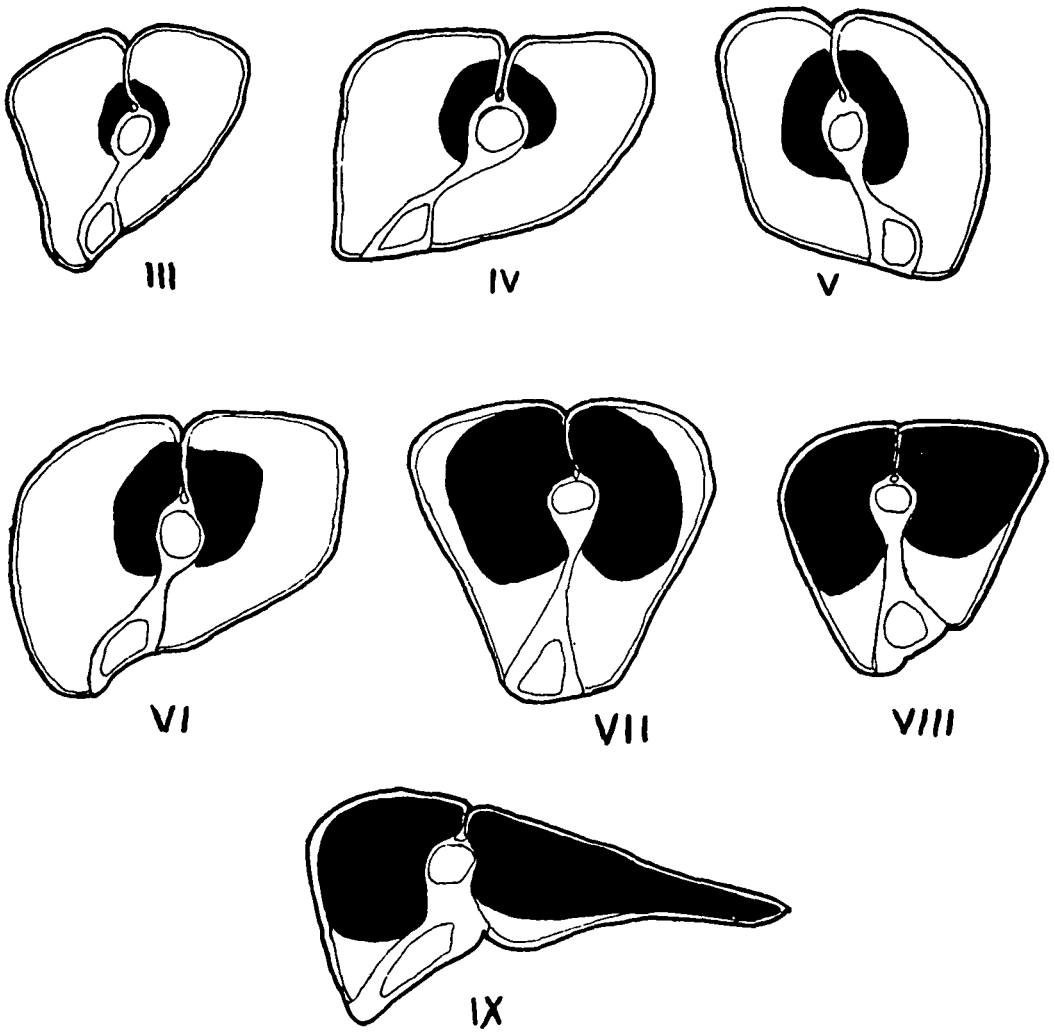

FIG. 6. Projection diagrams of the lamellae near the base of each of the major gills (gills III to IX). The area of the dense osmoregulatory tissue is indicated by solid black. See Figure 5 for a photograph of gill IX.

eas of osmoregulatory tissue. Projection diagrams were made (Fig. 6) and the area of osmoregulatory tissue for each gill lamella was measured by planimeter (Table 1). It was immediately obvious that the relative amount of tissue varies from gill to gill. Expressing the area of the osmoregulatory tissue as percent of the total lamellar respiratory surface area in each pair of gill lamellae (Table 1), it is clear that a sharp difference occurs between the three posterior gills and those more anterior. The difference between gill VI and gill VII is of the order of three in magnitude. (Note: The planimeter values are relative and not absolute.)

TABLE 1. *Areas in planimeter values (relative).*

Gill	Resp. surface	Osmoreg. tissue	Percent tissue
III	31.1	3.1	9
IV	46.9	6.3	13
V	48.4	10.6	21
VI	60.2	13.6	22
VII	56.1	33.8	60
VIII	37.2	29.6	79
IX	40.5	35.6	87

Fine structure of the osmoregulatory tissue. The osmoregulatory tissue is characterized by cells that have fine, sheet-like, protoplasmic extensions, or folds, that interdigitate with those of neighboring cells (Fig. 7). Spaces between the in-

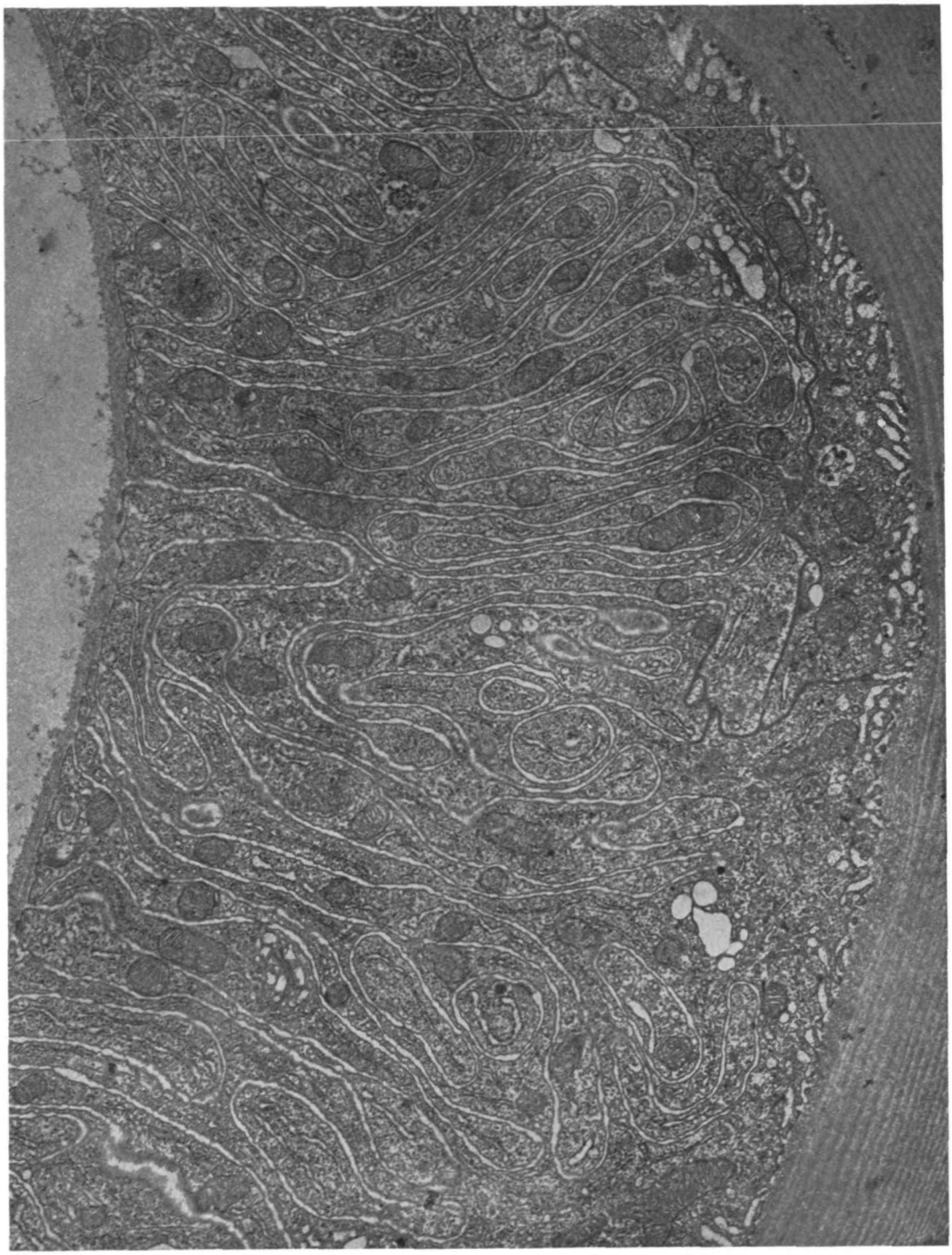

FIG. 7. Fine structure of osmoregulatory tissue. Cuticle at right. Plasma surface plus granular basement membrane at left. Extensive interdigitating folds of cells are seen. × 12,500

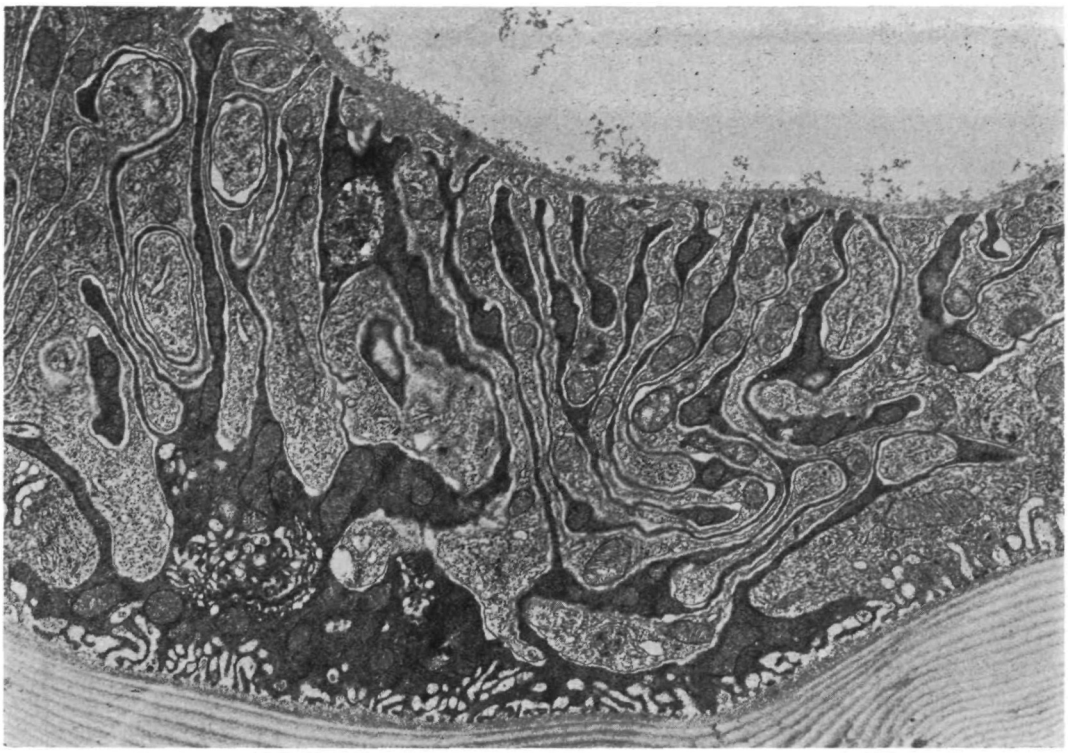

FIG. 8. Dark (moribund) cell. Note the regular, one-to-one alternation of folds interdigitated with those of a neighboring cell. × 10,000

terdigitating folds communicate freely with the plasma surface and its basement membrane. These spaces extend from the plasma surface close to, but never touching, the cuticular surface (Figs. 8 and 9). The distance between folds (*i.e.*, between cells) is variable (Fig. 10), but it is quite large compared to that in other tissues, 0.1μ being representative.

The folds are highly populated with mitochondria and, in their thicker parts, with rough endoplasmic reticulum oriented to the greater dimension of the folds. The interdigitation between cells is by very regular alternation, *i.e.*, it is rare that the fold from one cell will be positioned adjacent to another fold from the same cell. The occasional presence of a dark (moribund) cell allows the interdigitations to be traced with accuracy (Fig. 8).

The plasma surface and its associated basement membrane are sometimes thrown into a fold or a shallow groove. The interdigitating folds still reach these surfaces, resulting in a slightly different orientation of the folds (compare Figs. 7 and 9).

The cuticular surface of the cells is thrown into folds and tubular indentations (Fig. 9), much deeper than those seen subjacent to the cuticle in other parts of the body (Fig. 11). There are some indications of pinocytotic activity at the base of the folds (Figs. 7 and 9).

As stated above, the greater part of the intercellular surfaces is involved in the interdigitating processes. The parts of neighboring cells near the cuticle not involved in interdigitation are joined by septate desmosomes (Figs. 7 and 9).

One of the unusual and distinct features of the osmoregulatory tissue is the presence of "mitochondrial pumps" (Figs. 9 and 10). These have been described by me in three other animals (see

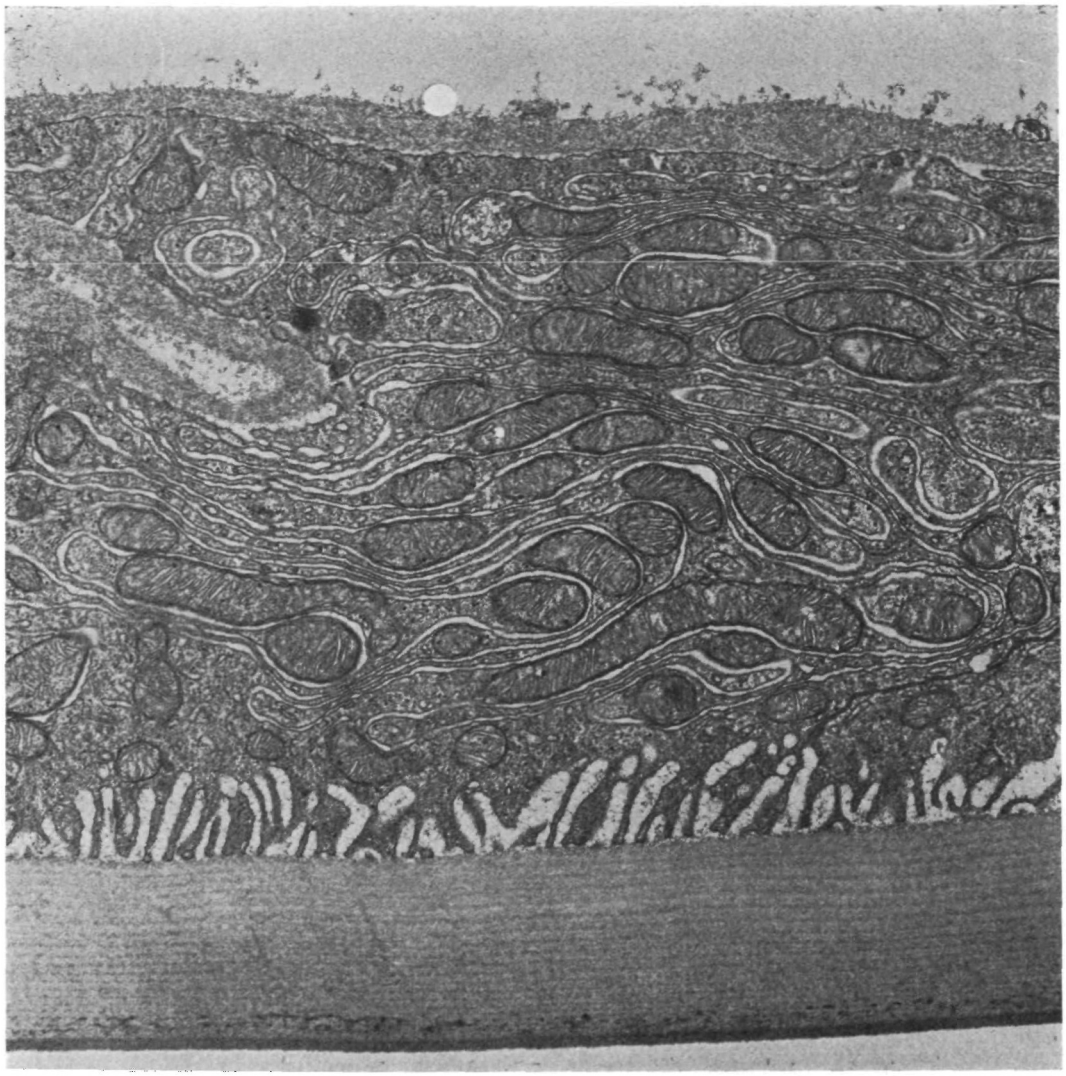

FIG. 9. Osmoregulatory tissue. Note the infolding of the basement membrane (left) and the orientation of the interdigitating folds (compare Fig. 7). Mitochondrial pumps are visible. × 17,000

discussion). They are defined as a close, parallel arrangement of plasma membranes and mitochondrial envelope-membranes with a spacing of 100-150 Å. The impression is gained that there may be a physical bonding between the plasma and mitochondrial membranes, which maintains the regularity of alignment (arrows in Fig. 10).

Fine structure of the pericardial sac. The cuticle of the sac is subtended by an epithelium of interdigitating cells (Figs. 11 and 12). However, unlike those of the osmoregulatory tissue, the interdigitating folds have no open space between them, possess few mitochondria, and do not reach the basement membrane (at the plasma surface) with regularity.

Figures 11 and 12 show interdigitating "light" and "dark" cells. The presence of light and dark cells is the exception rather than the rule, and they were chosen for these illustrations to demonstrate the interdigitations.

The basement membrane has openings or vesicles that are filled with fluid or other material having a light texture.

The surfaces of the cells next to the cuticle are thrown into shallow folds probably associated with secretion of cuticle. The sacs in Figures 11 and 12 came from an animal that was just beginning to secrete a new cuticle. Figure 12 shows an interesting cell that is probably one of the granular "reserve cells" described by Travis (1955) as being involved in secretion of cuticle.

The strands of muscle mentioned above (under gross morphology, Fig. 3) are composed of rather unusual muscle fibers, *i.e.*, in comparison to vertebrate muscle. This is striated muscle (Fig. 13) but with almost total lack of the bandings seen in the vertebrate skeletal muscle. Cross sections of the muscle fibers reveal larger myosin filaments together with the smaller actin filaments diagnostic of "striated" muscle (Fig. 14).

The muscle strands are anchored, where they abut the cuticle, by many clus-

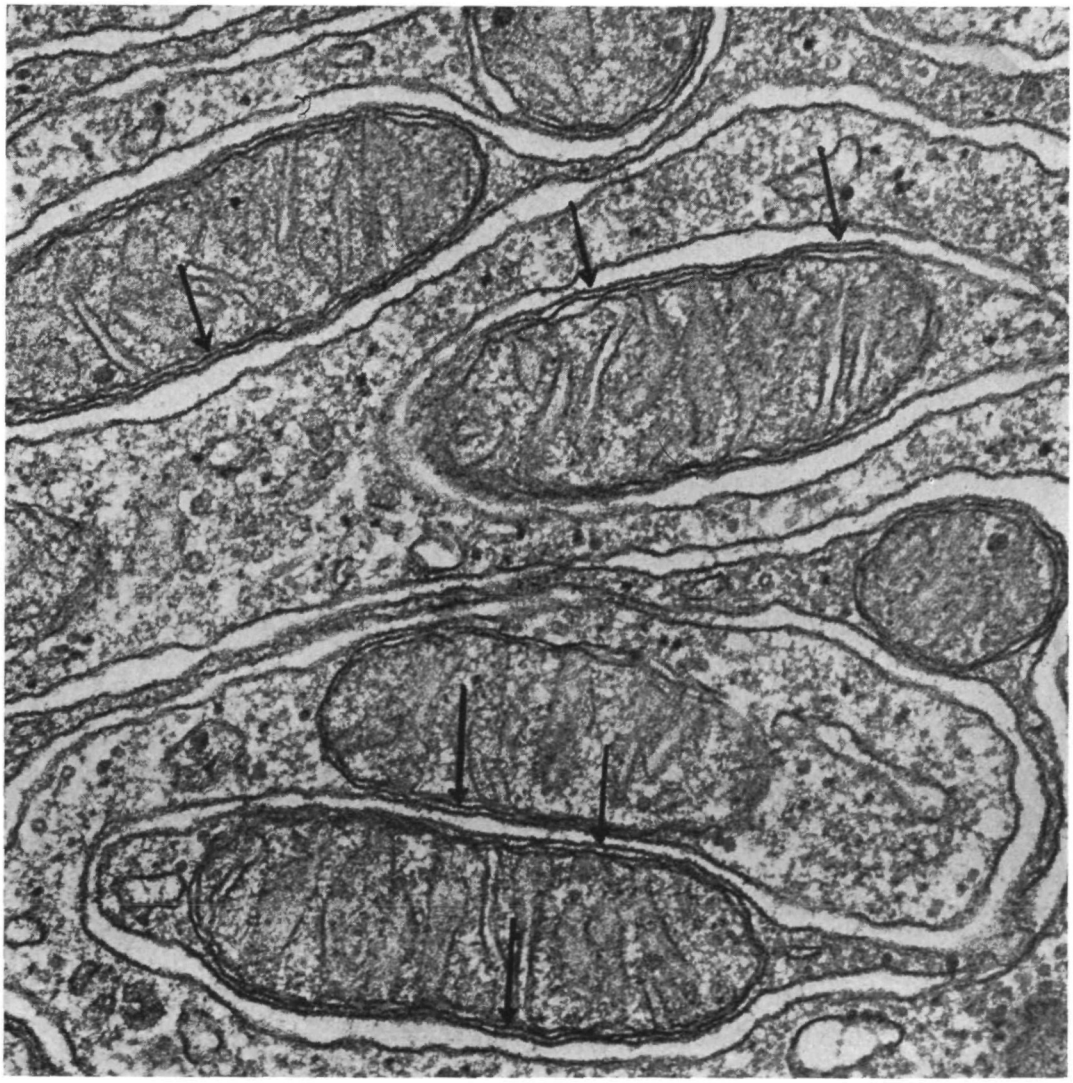

FIG. 10. Detail of mitochondrial pumps. Note the very regular spacing between the plasma membrane and the envelope membrane of the mitochondria (arrows). × 72,000

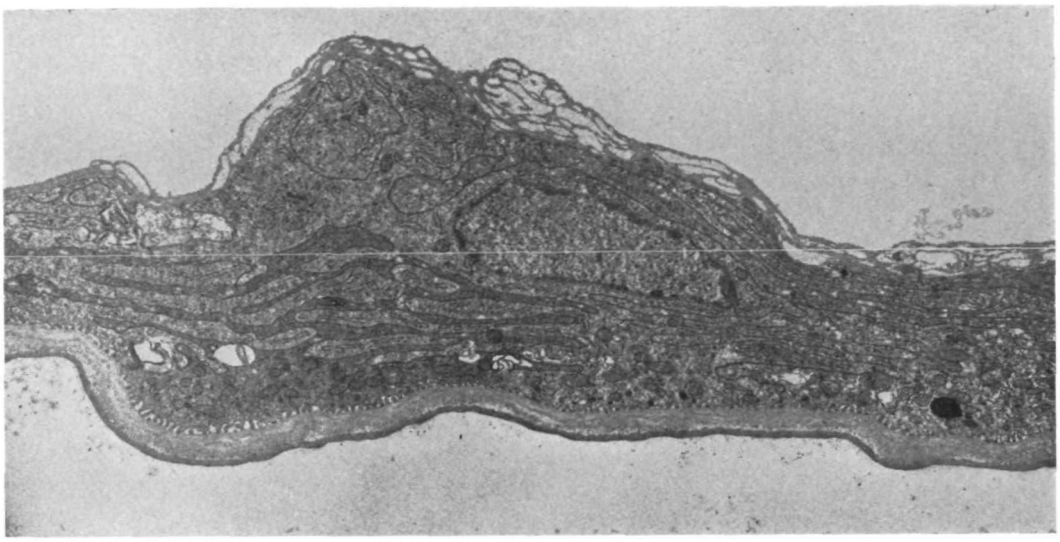

FIG. 11. Cuticular epithelium of the pericardial sac. Cuticle below. Vesicular basement membrane and plasma surface, above. × 6,250

ters of dark filaments that overlap the muscle filaments at one end and extend deep into the cuticle at the other end (Fig. 15). It would appear that the muscle strands of the pericardial sac are firmly anchored to the cuticular surface of the sac.

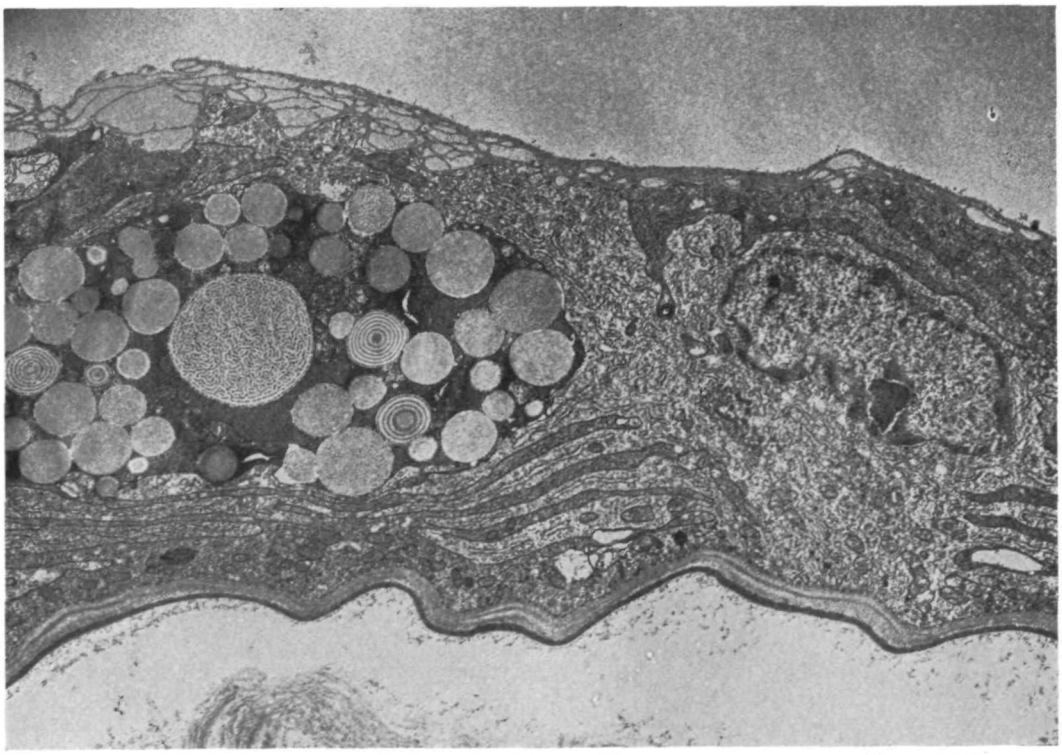

FIG. 12. Cuticular epithelium of the pericardial sac. Note granular "reserve" cell. × 7,500

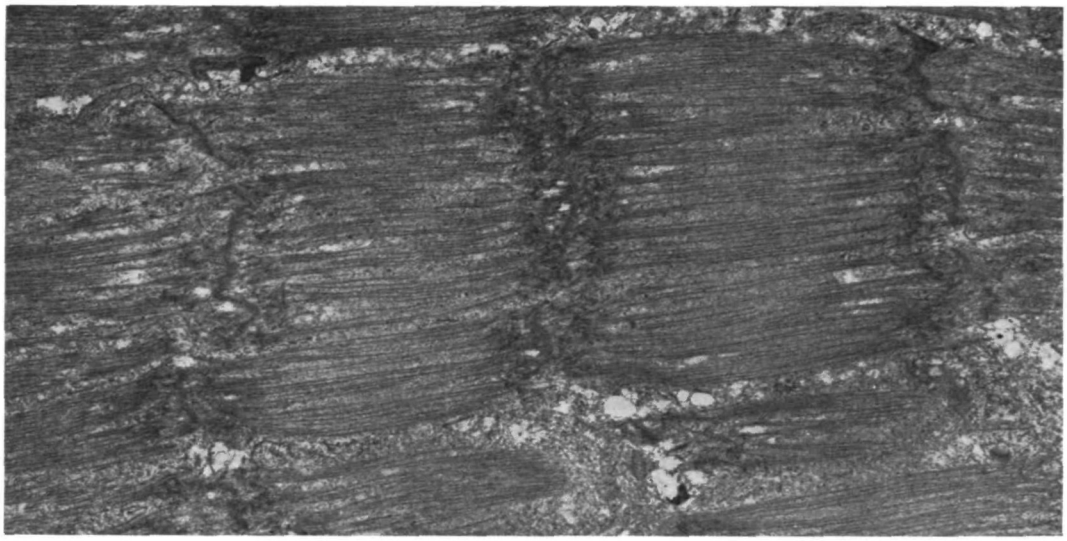

FIG. 13. Longitudinal section of muscle fibrils of the pericardial sac. Note the absence of H and M bands and the poorly defined Z line. × 6,300

DISCUSSION

The fine structure of the osmoregulatory tissue in *Gecarcinus* gills is almost identical to that in the gills of *Callinectes* (Copeland, 1968). One exception is that the extreme flattening of the mitochondria and the reorganization of the cristae seen in the mitochondrial pump of *Callinectes* were not observed in *Gecarcinus*. This may be due to the small number of *Gecarcinus* that were studied because of the difficulties of fixation.

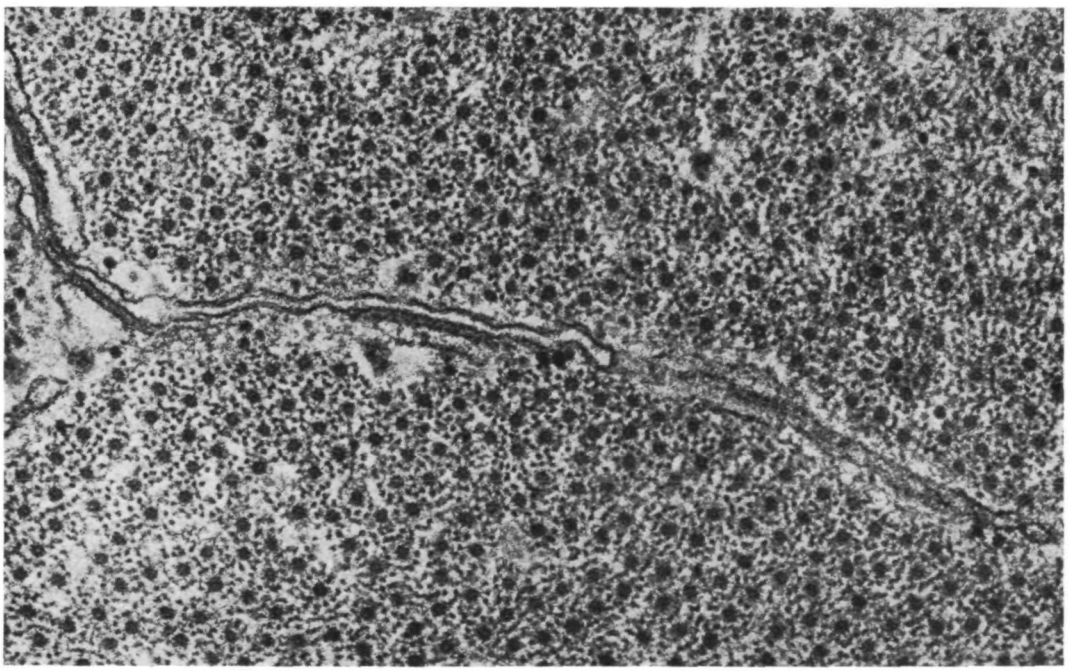

FIG. 14. Cross section of muscle fibrils of the pericardial sac. The large myosin filament cross sections are easily seen. The small actin filament cross sections are much more numerous. × 72,000

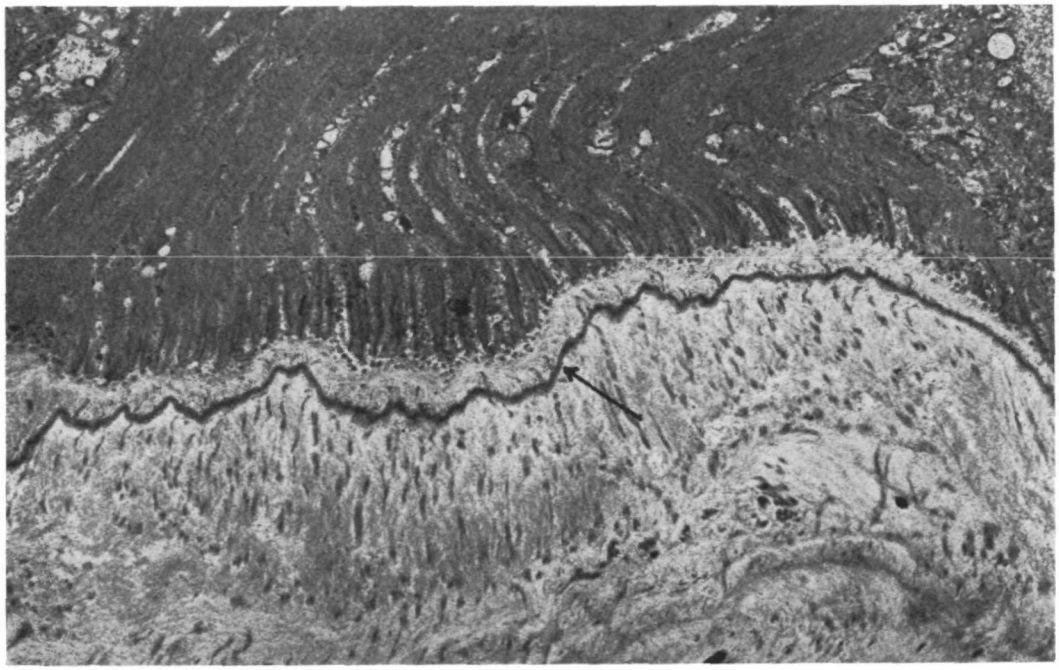

FIG. 15. Anchorage of the muscle strands in the pericardial sac to the cuticular surface of the sac. The dark line (arrow) represents the interface between the old cuticle and a new one just starting to be secreted prior to actual molt. × 10,000

There is no question, however, that the mitochondria in the *Gecarcinus* mitochondrial pumps tend to be flattened and pudding-shaped. The characteristic association of mitochondria and invaginated or infolded membranes at distances of the order of 100-150 Å is also seen. This membrane association has been identified in other salt-transporting tissues: anal papillae of mosquito larvae that can absorb salt from water of 0.009‰ total salinity (Copeland, 1964*a*), metepipodite segments of brine shrimp, which can secrete salt against saturated salt concentrations (Copeland, 1966, 1967); gills of blue crab, which can absorb salt when the crab adapts toward fresh water concentrations (Copeland, 1964*b*, 1968).

Two other instances of salt-transporting structures in the invertebrates should be mentioned. One is the highly ordered piles of plasma membrane folds associated with mitochondria in the rectal papillae of the blowfly. These are believed to actively absorb salt and passively absorb water (Gupta and Berridge, 1966; Berridge and Gupta, 1967). The other is the finger-like cytoplasmic protrusions, closely encasing mitochondria, in the "goblet" cells of the *Cecropia* midgut. These are thought to be associated more specifically with potassium transport (Anderson and Harvey, 1966) and differ from the above "pumps" in that there is a differentiated layer between the mitochondrial and plasma membranes. Furthermore, it is suggested that the mitochondrial membranes, rather than the plasma membranes, are the more likely site of the active transport of potassium.

On the basis of comparison with tissues in other organs known to transport salt in invertebrates, I suggest that the highly interdigitated and complex cells lining the respiratory lamellae of the *Gecarcinus* gill do indeed absorb salt, a physiological function known to reside specifically in the crab gill (Koch, *et al.*, 1954). Furthermore, the greatest share of the total salt

absorption occurs in the three most posterior gills, which rest on the surface of the pericardial sac.

I should also like to suggest that the cells responsible for absorption of salt do at the same time absorb water that, as shown by Bliss (1963; see also Bliss, *et al.*, 1966), has moved by capillary attraction from the ventral setae of the animal up to the surface of the pericardial sac.

At present there are two model systems under discussion that may explain the cellular mechanisms of water movement. One is the double membrane theory of Curran (1960, 1965) which has been applied to the structures observed in the rabbit gall bladder (Whitlock and Wheeler, 1964; Whitlock, *et al.*, 1965). The other model is the standing osmotic gradient theory of Diamond (1962, 1965). This has also been applied to interpretation of physiologically induced changes in the structure of the rabbit gall bladder (Diamond and Tormey, 1966).

As discussed in more detail in the report on the *Callinectes* gill (Copeland, 1968), I believe Diamond's model is more applicable to the channels or spaces between the interdigitating folds of the cells in the crab gill than is Curran's model (Curran and McIntosh, 1962). In either event, the channels between the cells of the crab gill show marked resemblances to the physiologically induced conditions in the gall bladder (above), as well as induced changes in the rectal papillae of the blowfly (Berridge and Gupta, 1967).

Attention is called to the data presented in Table 1. The three posterior gills, which cover the pericardial sac, have the highest ratio of osmoregulatory tissue per area of the respiratory lamellae. Figure 1 shows that the two most posterior gills (which have a ratio of 80% or higher) have respiratory lamellae that are spaced exceptionally close to each other. I suggest that these lamellae are close enough together to attract water from the surface of the pericardial sac in such a manner as to completely fill the inter-lamellar spaces with fluid. This would enhance the capability of these two gills to absorb salt actively from the water and, in turn, to absorb water passively. By the same reasoning, this circumstance would lower the capability of these gills to carry on respiration (which would be handicapped in any event by the presence of the thick osmoregulatory tissue lining the major surface areas of the respiratory lamellae).

The fine structure of the walls of the pericardial sacs as reported here does not suggest that water is absorbed through the sac walls as hypothesized by Bliss (1963). If it is agreed that water is rarely or perhaps never transported metabolically as an entity but is moved as an adjunct to active transport of other ions, then the observed structure of the sac epithelium fits none of the cellular mechanisms mentioned above. The tissue is interdigitated, but so are many epithelia. Furthermore, the interdigitations are not highly populated with mitochondria, do not communicate regularly to either surface of the epithelium, and do not have the intercellular spaces characteristic of active gall bladder.

Two reservations are made on the interpretation of structure as related to possible water absorption by the pericardial sac. One is the obvious possibility that the internum of the sac may have an osmotic pressure such as to passively absorb water. The second reservation relates to the fact that my observations were limited to the intermolt condition. That is, the condition of the sac during the rapid swelling just prior to molt was not checked (see discussion at the end of this session).

There is no question of the storage function of the pericardial sac as it swells prior to molt. A second function may also exist. The sac is bound together by many strands of muscle, crossing the lumen of the sac as well as forming a network parallel to the surface. This has been described at the level of the light microscope by Bliss (1963), and my observations with the electron miscroscope reveal a modified striated muscle similar to the

slow-acting type B muscle described by Reger (1967) in the crab, *Pinnixia*. These muscle strands are firmly anchored to the cuticle. To the above can be added an observation by Linda Habas Mantel (personal communication) that the pericardial sacs of *Gecarcinus* contract alternately during ecdysis. It is natural to suggest that, in addition to storage, the pericardial sacs act as hydraulic pumps and thereby help the crab to withdraw from its old shell.

In conclusion, I suggest that the water and salts attracted from damp sand to the surface of the pericardial sac are picked up by the posterior gills and the salt is actively absorbed; the water is passively absorbed as part of the procedure. The pericardial sac, during intermolt, serves as a flexible, contoured cushion maintaining close contact with the lower surfaces of the posterior gills, thereby supplying accumulated ground water to the respiratory lamellar spaces. At time of molt, the pericardial sac serves as a storage organ and as a hydraulic assist in shedding the old shell.

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