

TELEOST CHLORIDE CELL

I. Response of Pupfish *Cyprinodon variegatus*

Gill Na,K-ATPase and Chloride Cell Fine

Structure to Various High Salinity Environments

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ABSTRACT

Certain euryhaline teleosts can tolerate media of very high salinity, i.e. greater than that of seawater itself. The osmotic gradient across the integument of these fish is very high and the key to their survival appears to be the enhanced ability of the gill to excrete excess NaCl. These fish provide an opportunity to study morphological and biochemical aspects of transepithelial salt secretion under conditions of vastly different transport rates. Since the cellular site of gill salt excretion is believed to be the "chloride cell" of the branchial epithelium and since the enzyme Na,K-ATPase has been implicated in salt transport in this and other secretory tissues, we have focused our attention on the differences in chloride cell structure and gill ATPase activity in the variegated pupfish *Cyprinodon variegatus* adapted to half-strength seawater (50% SW), seawater (100% SW), or double-strength seawater (200% SW). The Na,K-ATPase activity in gill homogenates was 1.6 times greater in 100% SW fish than in 50% SW fish, and 3.9 times greater in 200% SW than in 100% SW. When 50% SW gills were compared to 100% SW gills, differences in chloride cell morphology were minimal. However, chloride cells from 200% SW displayed a marked hypertrophy and a striking increase in basal-lateral cell surface area. These results suggest that there are correlations among higher levels of osmotic stress, basal-lateral extensions of the cell surface, and the activity of the enzyme Na,K-ATPase.

Certain euryhaline teleosts can tolerate a remarkably wide range of external osmolalities (0.1–>2,300 mosmol/kg). The osmotic gradient across the integument of these fish is in most cases very high since their internal environment varies little, in the relatively small range of 250–500 mosmol/

kg (20, 21, 31). It is well known that the most important osmoregulatory organ in these animals is the gill, which is characterized by a relatively slow absorption of Na and Cl in low salinity environments, e.g., fresh water (FW), and a relatively rapid secretion of Na and Cl in high salinity envi-

ronments, e.g., seawater (SW). Recently, a great deal of attention has been focused on the enzyme Na,K-ATPase (ouabain-sensitive, sodium and potassium-dependent adenosine triphosphatase) in the gill and other specialized salt-transporting epithelia (2). The idea that this enzyme plays a fundamental role in transepithelial salt transport finds support in teleost gills since the activity of gill Na,K-ATPase, like Na flux, is several times higher in SW-adapted fish than in FW-adapted fish (for reviews see references 23 and 28). However, the directions of net sodium flux are opposite in fish adapted to these two environmental extremes. One approach to elucidating the underlying mechanism of gill Na transport is to focus attention on the role of gill Na,K-ATPase in those adaptive states characterized solely by net Na secretion. For example, physiological and biochemical studies have shown that net Na secretion is higher in 100% SW than in 40% SW (38, 39), and that gill Na,K-ATPase activity is higher in 100% SW than in 50% SW (3, 49).

The cellular basis for Na and Cl transport across the teleost gill epithelium is believed to be the chloride cell, so named because of its large, secretory-like appearance and the timely description (16) which immediately followed the first demonstration of active chloride excretion by the teleost gill (15). This cell shares several cytoarchitectural characteristics with transporting cells in other secretory epithelia (35), namely, a greatly amplified basal-lateral cell surface and a rich population of mitochondria. Furthermore, chloride cell morphology responds to changes in the salt concentration of the external environment: chloride cells of SW-adapted teleosts are either larger (1, 50) or both larger and more numerous (12, 17, 45, 49, 51) than chloride cells of FW-adapted teleosts. In fact, there is now convincing evidence that chloride cells possess most of the gill Na,K-ATPase. First, chloride cell-rich fractions isolated from dissociated gill epithelium exhibit many times the activity of gill cell fractions lacking chloride cells (13, 44). Second, [³H]ouabain autoradiographic data presented in the accompanying paper (14) provide direct evidence for chloride cell localization.

To study the relationship between gill Na,K-ATPase activity and chloride cell morphology, we chose for an experimental animal the euryhaline teleost *Cyprinodon variegatus*, a species which has been captured from natural environments having a salinity as high as four times that of seawater (11, 46). To focus exclusively on salt excretion by the

branchial epithelium, we examined gills of this fish adapted to the high salinity environments of 50%, 100%, and 200% SW. We have found that increases in Na,K-ATPase activity roughly parallel the proliferation of chloride cell basal-lateral cell surface membrane. The present study provides indirect support for the view that most Na,K-ATPase is located primarily on the tubular system membrane of the chloride cell. The accompanying paper (14), which describes the use of [³H]ouabain autoradiography to localize gill and chloride cell Na,K-ATPase, provides convincing support for this view.

MATERIALS AND METHODS

Animals

Specimens of the variegated pupfish *Cyprinodon variegatus* weighing 1.0–3.5 g were collected in coastal brackish water sloughs near Port Bolivar, Tex. during the months of March–August and December–February and stored in well water (ca. 80 mosmol/kg) for several days before transfer to a marine cold room (18–22°C) where the adaptations were carried out. All concentrations of artificial seawater (SW) were made with Utility Seven Seas Mix (Utility Chemical Company, Paterson, N. J.).

All fish were maintained in 100% SW for 3–5 days before the start of the adaptation schedule. Fish were adapted to the three environments of 50%, 100%, and 200% SW using two general approaches. In one approach, fish were transferred at 2- or 3-day intervals into freshly prepared environments of increasing or decreasing salinity (total of six transfers). Alternatively, fish remained in the same tanks and the adaptation environment was either diluted or concentrated in 24 proportional steps spaced 12 h apart. There were no differences in results with either method. Throughout the experiments fish were fed chopped calf liver or commercial dried fish food (TetraMarin:TetraWerke, Melle, West Germany).

Fish that had been in their respective experimental environments for 5.5 days or 10.5 days were weighed and pithed, and the gills were excised for fixation and/or assay of Na,K-ATPase activity. These adaptation times are reasonably close to the 7 days used for obtaining the gill Na flux measurements that have been published concerning 200% SW-adapted teleosts (24, 39). The second gill arch on the right side was used for fixation and the other seven arches were used for enzyme assay when this was measured. Excised gill arches were immediately immersed in either several drops of the fixative or in 0.25 M sucrose (0–5°C) for assay of ATPase activity.

Na,K-ATPase Assay

Gill arches were washed twice in 0.25 M sucrose and then homogenized in this medium (5 mg wet wt tissue/

ml). All gill homogenates were assayed for ATPase activity immediately after preparation. The assay system was a modification of that used by Ernst et al. (8). Homogenates were assayed at 37°C for both total ATPase activity (Medium A) and ouabain-insensitive ATPase (Mg-ATPase) activity (Medium B). The concentration of the components in both medium A and medium B was adjusted to give the following final concentrations in a 1.5-ml reaction mixture: 3 mM ATP (disodium salt from Sigma Chemical Co., St. Louis, Mo.), 3 mM MgCl₂, 5 mM KCl, 60 mM NaCl, and 92 mM Tris-(HCl) buffer, pH 7.2. Medium B contained, in addition, 0.1 mM ouabain. Ouabain-sensitive ATPase (Na,K-ATPase) activity was determined by the difference between the activity measured with Medium A and that measured with Medium B. Kinetic studies on gills from 200% SW-adapted fish showed that ATPase activity was proportional over a wide range of enzyme concentration and incubation time and that activity in the absence of Na or K was equivalent to that measured in the presence of these cations plus ouabain. Trichloroacetic acid-precipitable protein was determined by the method of Lowry et al. (18).

Microscope Studies

To lessen the possibility that morphological changes were caused by a particular fixation, several different procedures were employed. Before fixation, the gill arches were transected with razor blades so that individual pieces of the arch possessed several filaments. The pieces were then transferred to vials of fresh fixative. To enhance penetration of the fixative, the distal tips of the filaments were usually excised and discarded. Since this portion of the filament contains few chloride cells, it was always trimmed off before final sectioning. The three basic fixation schedules included the following: 3.25 h fixation at 0–5°C with 1% osmium tetroxide in phosphate buffer at pH 7.4 (27) containing 0.5 mM CaCl₂; 3.25 h fixation at 0–5°C in 2.5% glutaraldehyde (43) in phosphate buffer and postfixation for 90 min at 0–5°C in phosphate-buffered 1% osmium tetroxide; or 3.5 h fixation at room temperature with 6% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4 and postfixation for 90 min at 0–5°C in cacodylate-buffered 1% osmium tetroxide. After fixation, tissues were dehydrated with ethanol and embedded in Epon 812 (19).

Sections (0.8–1.2 μm) for light microscopy were cut with glass knives and stained with methylene blue and Azure II (42). Thin sections (500–800 Å) were cut with a diamond knife, stained for 5 min with 2% aqueous uranyl acetate adjusted to pH 5 with 1 N NaOH, and counterstained for 5 min with lead citrate (40). These thin sections were examined with an RCA EMU-3F electron microscope operated at 50 kV or a Philips 200 electron microscope operated at 60 kV. Morphological observations were carried out on at least 11 fish from each adaptation environment, including representatives from each of three complete experiments.

RESULTS

Adaptive Changes in Gill ATPase

Adaptation to higher salinities was accompanied by increases in the specific activity of gill Na,K-ATPase. As shown in Table I, the activity for 100% SW adaptation was 1.6 times that for 50% SW adaptation, and the value for 200% SW adaptation was 6.3 times that of the lowest salinity studied. In contrast, minimal increases were observed in gill Mg-ATPase activity. Only the 1.3-fold difference in activity between 50% SW adaptation and 200% SW adaptation was significant.

Morphology of Chloride Cells from 100% SW-Adapted *Cyprinodon variegatus*

The histology of the teleost gill and the cytology of the chloride cell are similar in many species of fishes (reviewed in reference 22) and will be treated only briefly here. Figs. 1 and 2 show the overall morphology of gill filaments and the distribution of chloride cells in the branchial epithelium of *Cyprinodon variegatus*. This cell type is predominantly located on the same side of the filament as the afferent branchial artery and between the bases of the respiratory leaflets. These large cells (8 μm in width and 15 μm in length) are usually columnar and exhibit a basal nucleus and a mitochondria-rich cytoplasm. The apical plasmalemma assumes the form of a cryptlike invagination with a diameter of about 2.5 μm (Figs. 2, 3). This morphological feature represents the hallmark of the 100% SW-adapted chloride cell. The crypt possesses an amorphous, polyanionic mucus (34, 36, 37). The cytoplasmic zone immediately adjacent to the crypt plasmalemma (Fig. 3) is characterized by the absence of mitochondria and

TABLE I
Gill ATPase Activities in *Cyprinodon variegatus*
Adapted to Various Salinities

Environment	N‡	Specific activity* (μmol Pi/mg protein × h)	
		Na,K-ATPase	Mg-ATPase
50% SW	12	4.08 ± 0.51	20.64 ± 1.15
100% SW	11	6.46 ± 0.79	23.49 ± 1.00
200% SW	11	25.49 ± 0.86	26.02 ± 0.82

* Values given as mean ± SE. Statistical comparisons: for Na,K-ATPase, 50% SW vs. 100% SW, $P < 0.02$; 50% SW vs. 200% SW, $P < 0.01$; 200% SW vs. 100% SW, $P < 0.01$; for Mg-ATPase, 200% SW vs. 50% SW, $P < 0.01$; other differences in Mg-ATPase activity not significant.

‡ N is the number of fish. Both 5.5-day (22 fish) and 10.5-day (12 fish) enzyme data are combined since there were no significant differences between values at these adaptation times.

tubular elements (see below) and by the presence of a dense population of secretory droplets. The fact that these secretory droplets are found in the Golgi complex and in the apical ectoplasm, as well as in the cytoplasm between these two regions, suggests a constant and heavy traffic of the droplets between the Golgi and the crypt. The situation here is perhaps analogous to the movement of vesicular-packaged glycoproteins in typical mucous cells (29).

A second prominent feature of the chloride cell distinguishes it from all other cell types in the gill. A major portion of the cytoplasmic ground substance is occupied by a branching tubular system which forms a characteristic three-dimensional reticular network. Horseradish peroxidase and lanthanum tracer studies have demonstrated convincingly that these tubules are invaginations of the cell surface and are not elements of the intracellular organelle, smooth endoplasmic reticulum (36). The configurations of this system are remarkably similar throughout the cytoplasm, and the network is excluded only from the Golgi complex and a narrow ectoplasmic zone beneath the apical crypt. It follows that the interstitial fluid inside the tubules is in intimate contact with a greatly extended cell plasmalemma and that the tubular ramifications of the cell surface are brought into a very close association with mitochondria and glycogen. These tubules are about 400 Å in diameter after osmium tetroxide fixation and about 600 Å in diameter after fixation in glutaraldehyde followed by osmium tetroxide.

The mitochondria of chloride cells are so large and numerous, compared to the mitochondria of other cell types in the gill, that they represent a third distinguishing chloride cell feature. Mitochondria are distributed throughout most of the cytoplasm except for Golgi regions and a narrow zone beneath the apical crypt (Fig. 3). They are rod shaped with a diameter of about 0.35 μm and a length of approximately 1.6 μm.

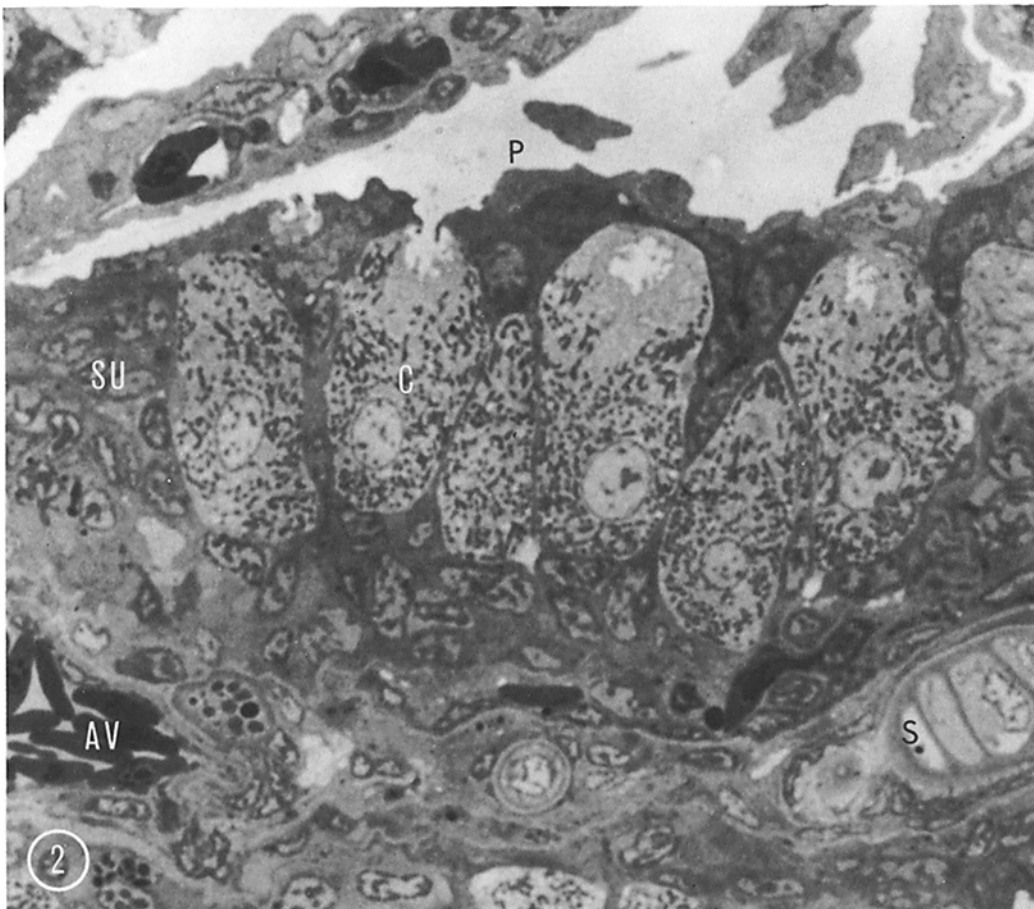
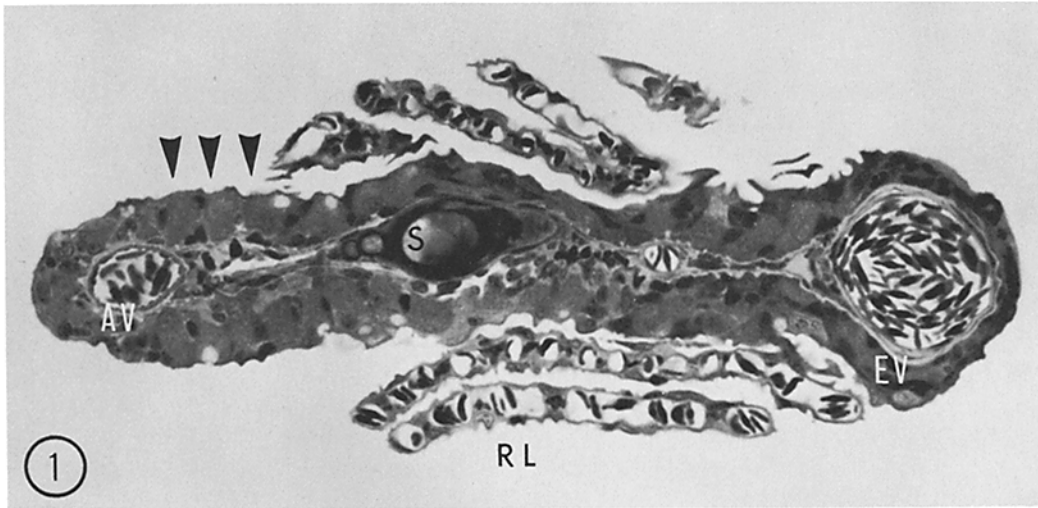
Chloride Cell Morphology after Adaptation to High Salinity Environments

Figs. 4–6 show representative light micrographs of transverse sections of gill filaments from fish adapted to the three environments of 50%, 100%, and 200% SW. In a survey of micrographs such as these, we observed that the chloride cell number was relatively constant in fish adapted to

the three environments. Chloride cells from 50% SW-adapted and 100% SW-adapted animals exhibited similar cell dimensions and fine structure. When light micrographs of gills from 200% SW-adapted fish are compared to those of gills of 100% SW-adapted fish, significant differences in cell size become apparent (Figs. 5, 6). Many of the 200% SW chloride cells were over twice as long as the 100% SW chloride cells, and maximal cell diameters were roughly 50% greater for chloride cells from the 200% SW environment compared to cells from fish adapted to the 50% or 100% SW environments.

In contrast to the similarity of chloride cell fine structure in 50% SW- and 100% SW-adapted animals, low magnification electron micrographs show that the tubular system is strikingly more conspicuous in the chloride cells of 200% SW-adapted (Fig. 8) than in the chloride cells of 100% SW-adapted fish (Fig. 7). This major difference can be seen to even greater advantage in Figs. 9 and 10. In the 200% SW-adapted fish, the chloride cell tubular extensions of the cell surface invade almost all of the available space not occupied by mitochondria. An estimation of the diameter of tubules from images showing cross-section profiles of them from 100% SW- and 200% SW-adapted fish reveals that the diameters are constant. Mitochondria appear to be noticeably smaller in the 200% SW chloride cell (compare Figs. 7 and 8). Mitochondrial counts taken from larger fields than those presented in the high magnification micrographs (Figs. 9, 10) also revealed that mitochondria are more densely packed in 200% SW chloride cells as compared to 100% SW chloride cells.

The present study also provided an opportunity to examine ultrastructural features which might reveal a correlation between salt excretion and mucus secretion. Specifically, careful attention was given to the fine structure of the Golgi complex, since it is known that this organelle plays a key role in the synthesis and/or packaging of mucopolysaccharide and/or glycoprotein secretory products in cells specialized for mucus secretion (29, 33). We observed no obvious differences to indicate that the Golgi complex was more active in any one environment or that the number of apical ectoplasmic secretory droplets varied with osmotic stress. Due to the large variation in preservation of the crypt mucus observed for any given adaptation environment, it was not possible to estimate the rate of mucus secretion on the basis of the quantity



of crypt mucus. Since electron micrographs are a static representation of dynamic events, it might prove interesting to utilize a technique such as autoradiography with tritiated monosaccharides as precursors, to explore further the relationship between mucus secretion and Na excretion in the gills of euryhaline teleosts adapted to various salinities.

It is highly unlikely that the observed morphological changes were due to the fixatives rather than osmotic stress. Because of the evidence, at least for some tissues, that aldehyde fixatives can cause cell shrinkage and/or swelling (10, 26, 48), several workers have used *both* an osmium tetroxide fixation *and* a glutaraldehyde-osmium tetroxide fixation (30, 48). In these latter studies, one on an absorptive epithelium, the gallbladder (48), and the other on a secretory epithelium, the insect salivary gland (30), both fixatives gave similar results. In the present study, consistent morphological results were obtained with the osmium tetroxide and glutaraldehyde fixation procedures employed.

DISCUSSION

Previous studies have shown that gills of euryhaline teleosts exposed to hyperosmotic environments, i.e. those greater than about 33% SW, exhibit increasing net Na secretory rates with adaptation to increasing salinities (38, 39). It is important to note that net Na secretory rate is much higher in 200% SW than in 100% SW (24, 39). In the present study, we have attempted to correlate

these increases in Na transport with alterations in the Na,K-ATPase activity, histology, and ultrastructure of the gill. Typically, differences between 50% SW and 100% SW gills were small. The roughly 60% increase in the specific activity of Na,K-ATPase between 50% SW and 100% SW may be too small to be resolved solely by fine structure morphology. In contrast, when compared with gills of 100% SW-adapted fish, those from 200% SW-adapted fish exhibited a fourfold increase in Na,K-ATPase activity, chloride cell hypertrophy, and proliferation of the basal-lateral (tubular system) membrane. We observed no changes in the number and size of chloride cell apical crypts in fish adapted to the three experimental environments. The salinity-induced hypertrophy of chloride cells in environments more concentrated than SW has been previously reported in three other euryhaline teleosts (light microscope observations: 1, 49, 50). Moreover, similar enzymatic and morphological adaptive changes between 100% SW and 200% SW were also observed by Karnaky et al. (14) in gills of the euryhaline killifish *Fundulus heteroclitus*. Using [³H]ouabain autoradiography, these workers demonstrated that gill Na,K-ATPase is located primarily on the amplified basal-lateral (tubular system) membrane of chloride cells and suggested that adaptive changes in enzyme activity reflect the quantity of tubular-system membrane. Certainly, the present morphological observations on the gills of *Cyprinodon variegatus* strongly support this view.

FIGURE 1 *C. variegatus* has four complete gill arches on each side of the body. This figure shows a cross section of one of the numerous gill filaments which project from each arch. The core of the filament consists of a cartilaginous spine (S), a small afferent blood vessel (AV), a larger efferent blood vessel (EV), and other connective tissue elements. The central canal or central sinus described in several teleost species (41, 47) was not observed in the gills of *C. variegatus*. Several respiratory leaflets (RL) are seen in cross section. Details of the branchial epithelium (region beneath arrowheads) are seen to a greater advantage in Fig. 2. 1 μ m section of gill filament from 100% SW-adapted fish, fixed with glutaraldehyde (cacodylate buffer). \times 600.

FIGURE 2 A small portion of a gill filament, again cut in cross section, near the afferent blood vessel (AV) and cartilaginous spine (S). A small portion of a respiratory leaflet is seen at the upper left side of the micrograph. The branchial epithelium surrounding the core of the filament consists of four major cell types: pavement (P), mucous (not shown), supporting (SU), and chloride (C). The pavement cells form the main covering of this epithelium and are absent only in those locations where the apical tips of chloride cells and mucous cells are directly exposed to the external aqueous environment. One of the chloride cells displays a prominent apical crypt, while two other chloride cells display apical "vesicles" because the opening of the crypt to the external environment is out of the plane of the section. Note that the mitochondria fill the cytoplasm except in the region beneath the apical crypt. 1 μ m section of gill filament from 100% SW-adapted fish, fixed with glutaraldehyde (phosphate buffer). \times 2,200.

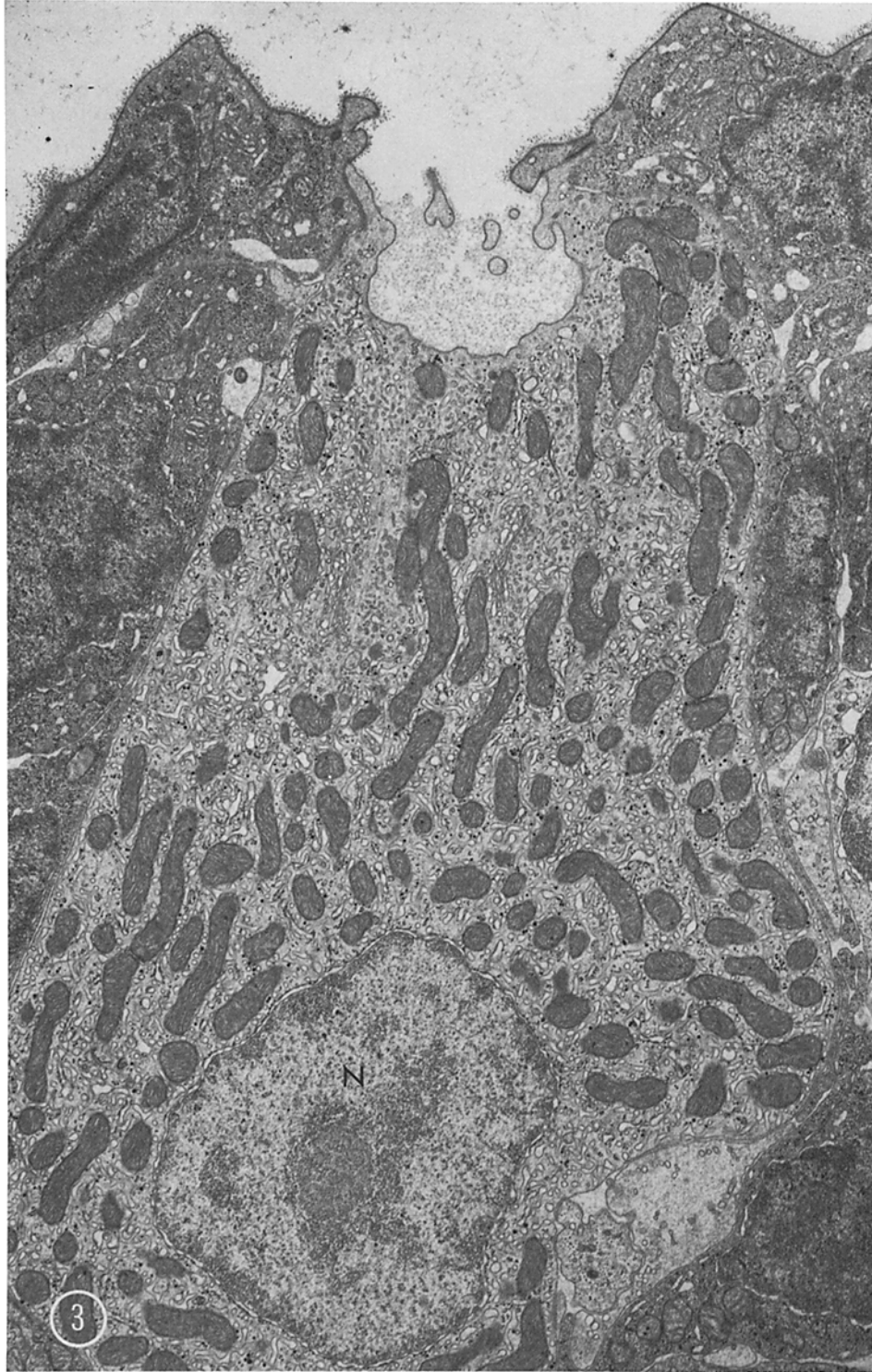
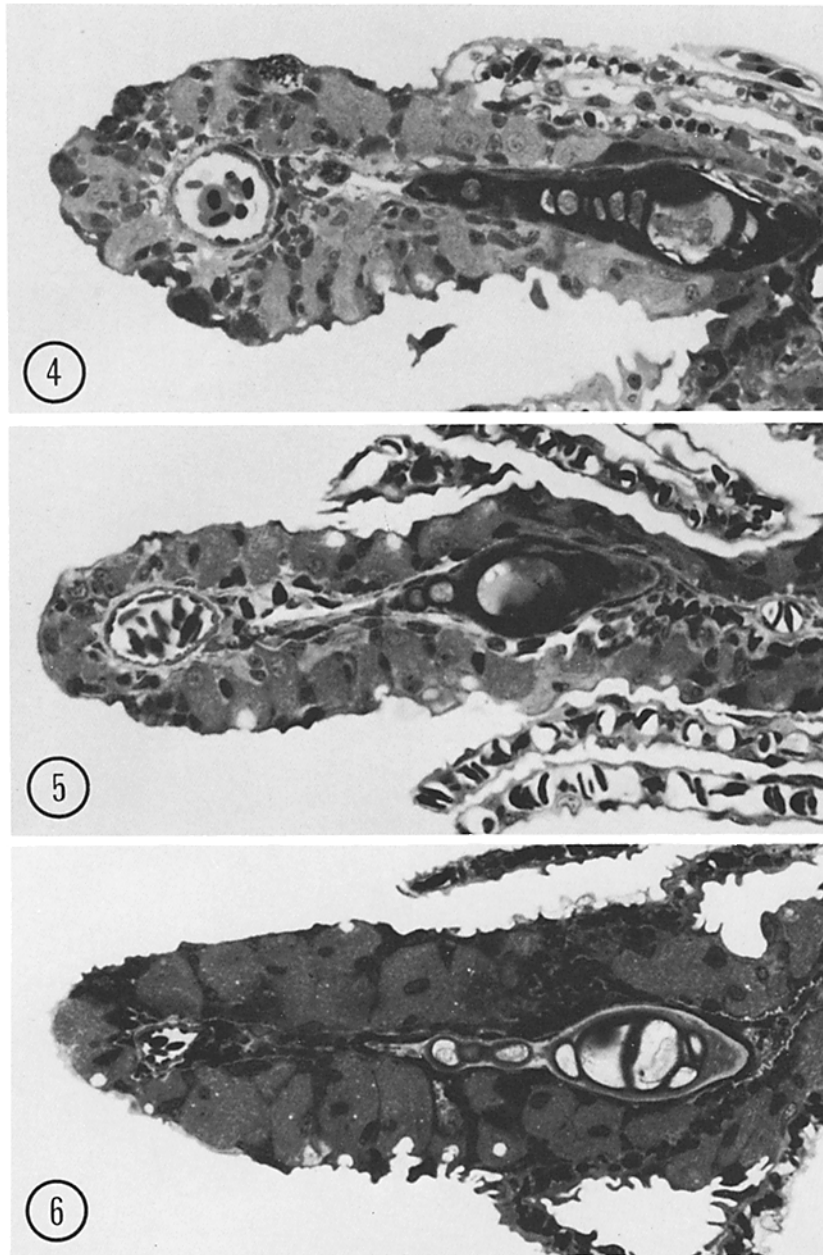
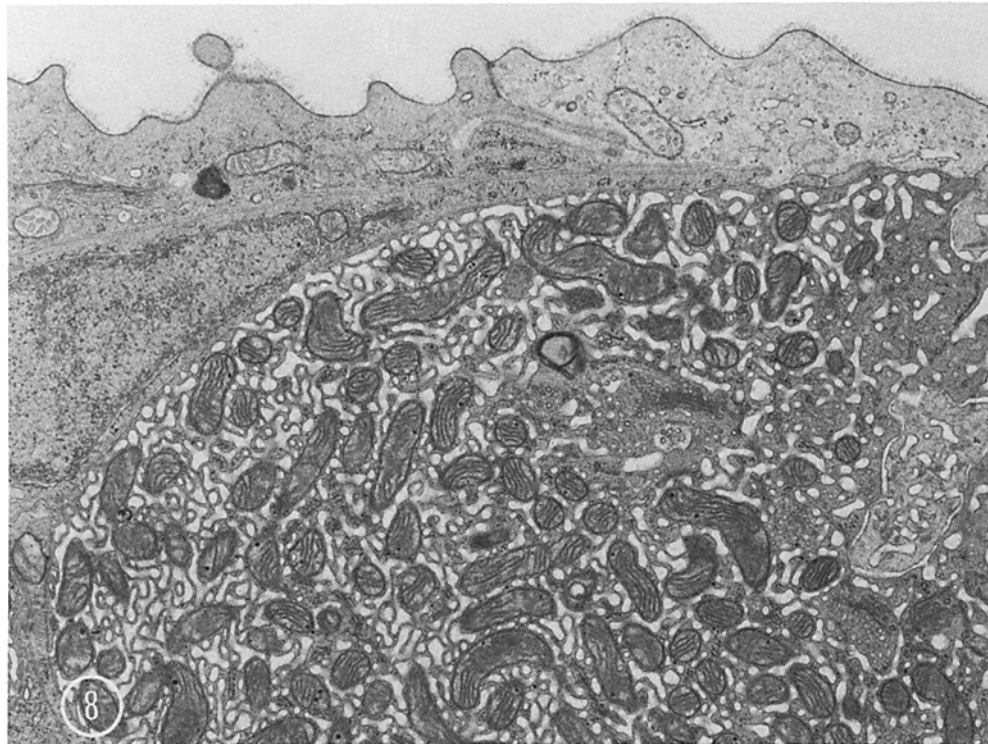
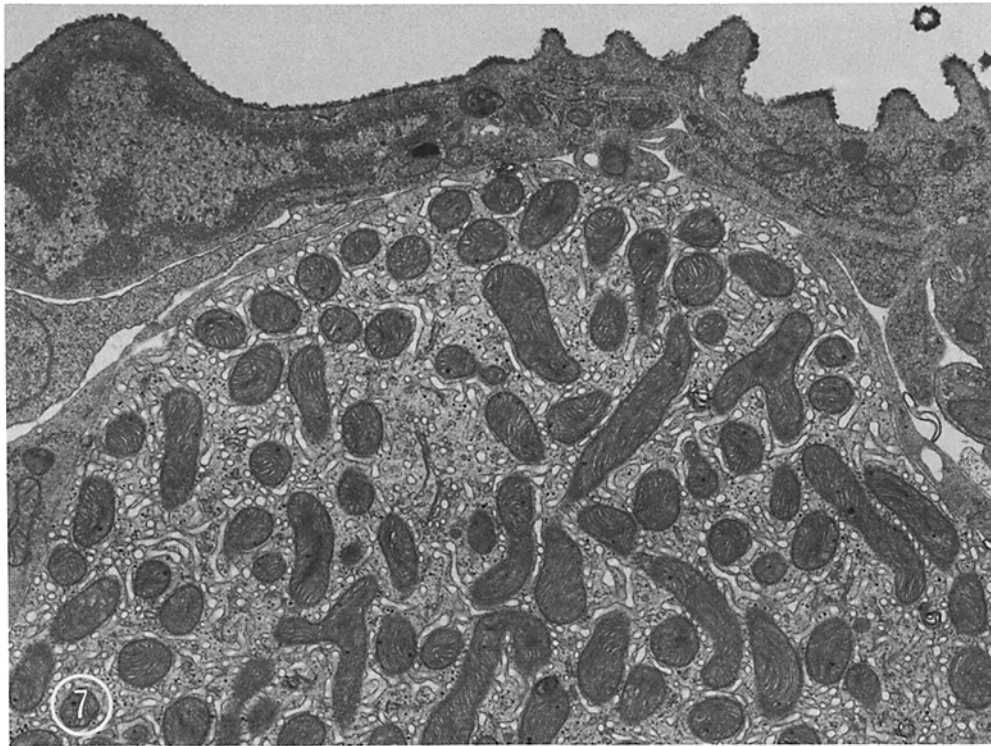


FIGURE 3 In this relatively low magnification electron micrograph, almost the full length of a chloride cell is depicted in longitudinal section. Small portions of the cytoplasm of two pavement cells are visible at the top of the micrograph. Note the large population of mitochondria. Golgi saccules can be identified in two areas of the supranuclear region of this cell. The apical surface of the cell invaginates to form the typical apical crypt. Note the crypt's complement of amorphous material (mucus). Numerous secretory droplets are located around the Golgi saccules, in the apical ectoplasm, as well as in the cytoplasm between these two regions. A portion of the nucleus (*N*) can be seen at the bottom of this image. Fixed with osmium tetroxide. Fish adapted to 100% SW. $\times 11,000$.



FIGURES 4, 5, and 6 Representative sections from gill filaments of specimens adapted to the three environmental salinities of 50% SW (Fig. 4), 100% SW (Fig. 5), and 200% SW (Fig. 6) are presented here at the same magnification and orientation. The afferent blood vessels are located at the left-hand side of the micrograph. The number of chloride cells appears to be roughly equal in the three environments. Although there appears to be little difference in the size of chloride cells in the gills of 50% SW-adapted and 100% SW-adapted animals, the chloride cells in gill filaments of 200% SW-adapted specimens are usually both wider and longer. Diameters of apical crypts, however, remain approximately the same. 1 μm sections of gill filaments fixed with glutaraldehyde (cacodylate buffer). $\times 750$.



The present results with the chloride cell of *C. variegatus* are similar to observations made on the avian nasal salt gland, an osmoregulatory salt-secreting tissue (32) whose secretion rate can be manipulated experimentally via dietary NaCl loading. The salt-stressed gland exhibits a parallel increase in the rate of secretion, the specific activity of Na,K-ATPase, and the development of highly specialized secretory cells which are characterized by extensive amplification of the basal-lateral cell surface by means of basal folds and lateral plications (7-9, 25). Recently, this basal-lateral cell surface was shown to be the site of the K-dependent, ouabain-sensitive phosphatase component of the Na,K-ATPase enzyme complex (5, 6). In the companion paper (14), the implications of the basal-lateral location of Na,K-ATPase are discussed in relation to the physiology of NaCl excretion by the teleost gill and other salt-secretory tissues.

It has been postulated that the surface extensions which characterize salt-secretory cells actually form long narrow channels in which geometrical parameters and transport site location play important roles in the coupling of water transport to active solute transport (4). Thus, an interesting question arises as to whether there is a special significance to the close packing of tubules in the 200% SW chloride cells of *C. variegatus*. Observations on the response to 200% SW exposure of chloride cells from *F. heteroclitus* (14) may bear on this question. The fact that, in this species, a presumed increase in the rate of excretion by chloride cells in 200% SW appeared to be accompanied by an increase in basal-lateral (tubular system) surface area mainly by means of cell hypertrophy, and only to a small extent by closer packing of tubules, suggests that surface area increase per se is the more important factor.

Finally, it may be that the ability of *C. variegatus*

to increase gill Na,K-ATPase activity to such a high level is related to the unique capability of this species to withstand certain waters with a salinity much greater than SW, such as occur along the Texas Gulf Coast (46). This may help explain in part the claim that *C. variegatus* is the "toughest" aquatic animal in North America (11).

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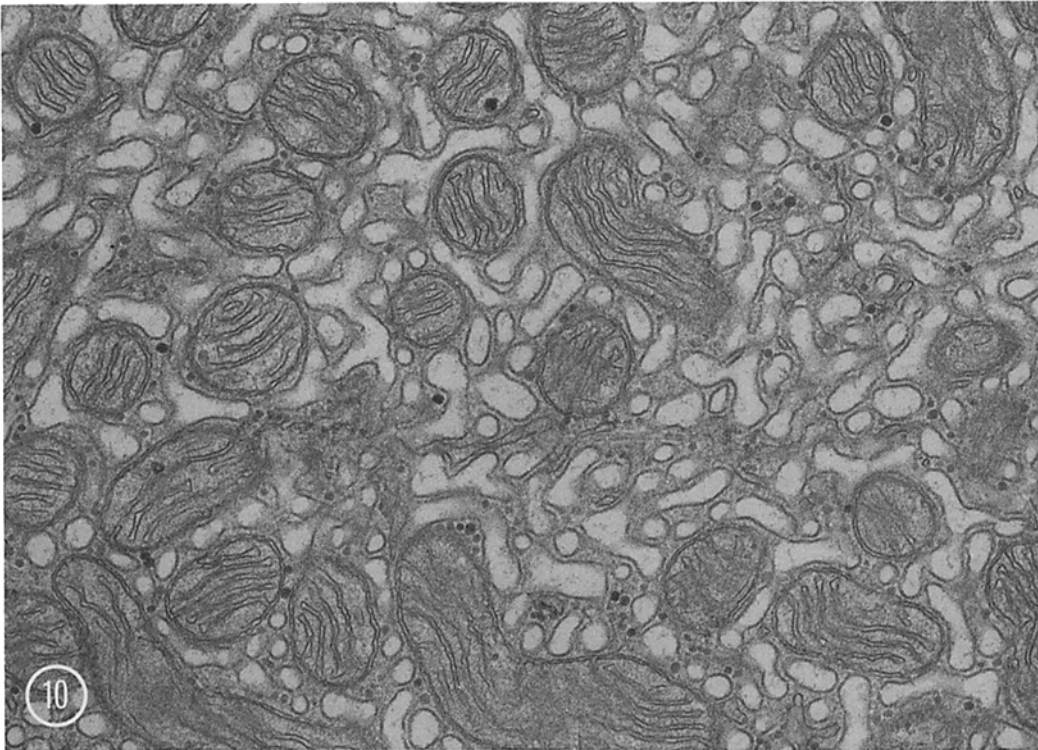
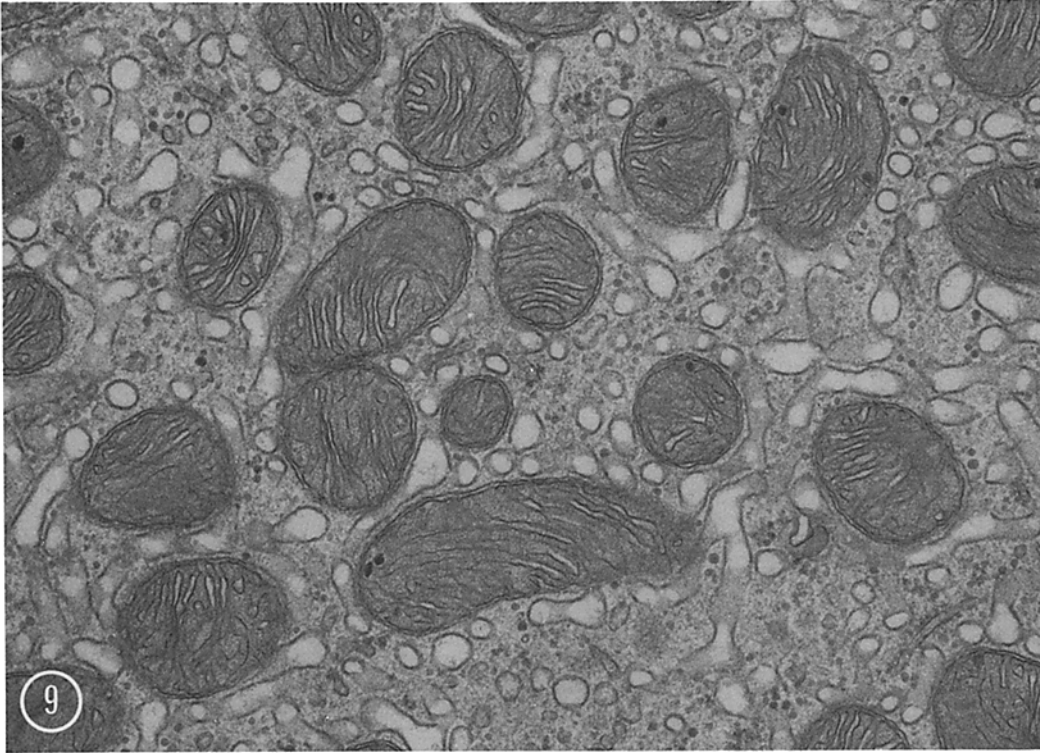
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FIGURE 7 An electron micrograph of a typical chloride cell from a 100% SW-adapted specimen. In this plane of section, the chloride cell is overlaid by squamous pavement cells. Note the extensive, branching tubular system which occupies a large portion of the cytoplasm. Note also the large population of mitochondria. Fixed with glutaraldehyde (cacodylate buffer). $\times 14,500$.

FIGURE 8 A representative chloride cell from a 200% SW-adapted fish. This micrograph is to be compared with the micrograph of a typical chloride cell from a 100% SW-adapted specimen, Fig. 7. In each case, section thickness and magnification are comparable. There are two major changes in chloride cell fine structure in 200% SW. First, the tubular system appears to occupy a much larger proportion of the cytoplasm than it did in 100% SW chloride cells. Second, mitochondria are smaller and more numerous in 200% SW. Fixed with glutaraldehyde (cacodylate buffer). $\times 14,500$.



FIGURES 9 and 10 The two major differences in the fine structure of chloride cells from the gills of 100% SW-adapted (Fig. 9) and 200% SW-adapted (Fig. 10) animals are shown to better advantage in these higher power views. Regardless of adaptation salinity, dense granules, apparently beta glycogen, are dispersed rather uniformly throughout the cytoplasm in which the tubular system and mitochondria are embedded. Fixed with glutaraldehyde (cacodylate buffer). $\times 43,500$.

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