# THE SURFACE EPITHELIUM OF TELEOSTEAN FISH GILLS

## Cellular and Junctional Adaptations of the Chloride Cell in Relation to Salt Adaptation

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ABSTRACT

Various species of teleostean fishes were adapted to fresh or salt water and their gill surface epithelium was examined using several techniques of electron microscopy.

In both fresh and salt water the branchial epithelium is mostly covered by flat respiratory cells. They are characterized by unusual outer membrane fracture faces containing intramembranous particles and pits in various stages of ordered aggregation. Freeze fracture studies showed that the tight junctions between respiratory cells are made of several interconnecting strands, probably representing high resistance junctions. The organization of intramembranous elements and the morphological characteristics of the junctions do not vary in relation to the external salinity. Towards the base of the secondary gill lamellae, the layer of respiratory cells is interrupted by mitochondria-rich cells ("chloride cells"), also linked to respiratory cells by multistranded junctions.

There is a fundamental reorganization of the chloride cells associated with salt water adaptation. In salt water young adjacent chloride cells send interdigitations into preexisting chloride cells. The apex of the seawater chloride cell is therefore part of a mosaic of sister cells linked to surrounding respiratory cells by multistranded junctions. The chloride cells are linked to each other by shallow junctions made of only one strand and permeable to lanthanum. It is therefore suggested that salt water adaptation triggers a cellular reorganization of the epithelium in such a way that leaky junctions (a low resistance pathway) appear at the apex of the chloride cells.

Chloride cells are characterized by an extensive tubular reticulum which is an extension of the basolateral plasma membrane. It is made of repeating units and is the site of numerous ion pumps. The presence of shallow junctions in sea water-adapted fish makes it possible for the reticulum to contact the external milieu. In contrast in the freshwater-adapted fish the chloride cell's tubular reticulum is separated by deep apical junctions from the external environment.

Based on these observations we discuss how solutes could transfer across the epithelium.

† Deceased 16 August 1977.



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How teleost fish adapt to both fresh water and salt water and yet maintain an internal milieu of constant osmolarity has been the subject of much biological curiosity. Many years ago Smith (51) and Krogh (22) showed that in salt water the teleost excretes NaCl through the gill to eliminate what it absorbs by ingestion, whereas in freshwater the fish drinks little and absorbs salt through its gill from the dilute environment. More recent work on the mechanisms of ion exchange across the gill has been reviewed (25). In addition to its role in osmoregulation, the gill has some other vital functions: exchange of respiratory gases (15), regulation of acid-base balance (13), excretion of small organic molecules (30, 31).

Flat, thin respiratory cells cover most of the gill epithelial surface, a smaller number of mucous cells and "mitochondria rich cells" make up the rest of the epithelium (references 6, 36, 44; see Fig. 1). Since chloride was localized in the "mitochondria-rich cells" (chloride cells), this cell type has been considered the site of excretion in salt water (21, 37). They may also perform salt absorption in freshwater (25).

For the epithelium, pumping salt in or out depending on the salinity of the external milieu represents an adaptative challenge that must be reflected in the organization and/or ultrastructure of its cells. Until now, adaptation to salt water has only been characterized by an increase in the number of chloride cells and in the development of their tubular reticulum (18, 48). These changes probably account for the increase in Na-K activated ATPase activity and ouabain binding sites of the gill tissue upon salt adaptation (11, 17, 19, 46). These modifications alone cannot explain satisfactorily why ions are absorbed in fresh water and excreted in salt water (for a discussion of this point see reference 19). Neither do they provide an explanation for the greatly increased salt permeability of the gill epithelium in salt water adapted fish (25).

We have examined the gill epithelium of several species of teleost fish adapted to fresh and salt water environments using freeze-fracture, thin and thick section electron microscopy. In this report we give a detailed description of how the gill surface is organized, examining the different cell types and the junctions that link them. We show that salt adaptation involves the development of young chloride cells which send arms into preexisting chloride cells. As these arms are terminated by leaky junctions, we believe the cells cooperate to excrete salt. We discuss this finding in relation to observations made on other salt-excreting epithelia. In addition, we describe newly observed features of the chloride cell and attempt to relate its structure to its function in fresh or salt water environment.[1]

## MATERIALS AND METHODS

Most of our observations were done on mullets (*Mugil capito*) or eels (*Anguilla anguilla*), but we also examined perfused trout heads (*Salmo gairdneri*), seawater adapted killifish (*Fundulus heteroclitus*) and guppy (*Lebistes reticulatus*). The fish were adapted for at least 3 wk in fresh water, salt water, or 200% artificial sea water (mullets and eels only). The gills were quickly dissected, fixed and processed for electron microscopy.

### *Thin-Section Electron Microscopy*

Gills were fixed 1 h at room temperature in glutaraldehyde (1.5-2% made from 8% glutaraldehyde from Polysciences, Inc., Warrington, Penn., or 25% glutaraldehyde from TAAB Laboratories, Emmer Green, Reading, England) in Na cacodylate buffer, pH 7.8, (0.05 or 0.08 M) so as to match the osmolarity of the fish plasma in different milieu. Some fixations were done with 0.4% glutaraldehyde in 0.08% Na cacodylate buffer with added sucrose (5%) and $CaCl_2$ (5 mM). Small pieces of tissue were treated with osmium (1% osmium (Merck Chemical Div., Merck & Co., Rahway, N. J.) in Na cacodylate) for 1 h in the cold and occasionally with mordant (1% tannic acid, Mallinckrodt, Inc., St. Louis, Mo.) (49). Dehydration was done in alcohol and embedding in Epon 812. Thin sections were poststained with uranyl acetate and lead citrate.

### *Thick-Section Electron Microscopy*

Pieces of gills from mullets and guppies were fixed in glutaraldehyde (1.5-1.8%) in Na cacodylate buffer, pH 7.8, (0.05-0.08 M). They were subsequently impregnated with aqueous uranyl acetate, poststained in double lead and copper citrate solution and overnight in osmium as described by Thiery and Rambourg (52), and embedded in Epon. Sections 0.5-1 $\mu$m thick were cut and examined in the electron microscope (Philips EM 300 at 100 KV).

### *Cytochemical Methods*

Colloidal Thorium (40) and the periodic acid-chromic acid-silver methenamine (41) staining technique were

[1] The present work was presented in poster form at the Societé Française de Microscopie, Annual Meeting in Nice May 31-June 1977. *Biologie Cellulaire* (1977), **29:** 25*a*. (Abstr.).

used on small pieces of gill tissue as described previously (38).

### *Experiments with Lanthanum*

The live fish (guppy or mullet) was immersed 15 min in a 1.5% solution of lanthanum nitrate dissolved either in distilled water or in a 500 mM NaCl solution isosmotic to sea water. Just before use, this transfer medium was alkalinized to pH 7.8 with NaOH, avoiding precipitation of La hydroxide (42). The animals were pithed and the gills were rapidly excised and fixed in glutaraldehyde in cacodylate buffer for 15 min, or 1% osmium for 40 min. After fast dehydration in ethanol, the gill fragments were embedded in Epon 812. Fixatives and dehydrating solutions were free of lanthanum. Thin sections were examined unstained.

### *Freeze-Fracture Electron Microscopy*

Gill arches fixed in glutaraldehyde as above were infiltrated (10–60 h) with 25–30% glycerol in fixative or cacodylate buffer in the cold. Small pieces were quickly frozen on gold stubs in Freon 22. Freeze-fracture was done on Balzers machines equipped with a 4 specimen holder device (−125°C) or double replica accessory (−150°C). Pt and C were evaporated on the freshly cleaved surfaces and the replicas were cleaned of organic materials with sulfochromic acid.

### *Scanning Electron Microscopy*

Sea water eel gills were fixed in glutaraldehyde and osmium as above, dehydrated in alcohol and amyl acetate and critically point-dried using liquid $CO_2$. The specimens were coated with gold in a Leybold evaporator (Leybold-Heraeus Vacuum Products Inc., Monroeville, Pa.) and examined in a Coates and Welter Low Emission Scanning Electron Microscope.

### *Negative Staining*

Chloride cells from eel gills were prepared as described (46). The cells were shattered in Hank's solution by two or three passes of pestle in a Dounce homogenizer (Kontes Co., Vineland, N. J.). A drop of the homogenate was deposited on a formvar carbon coated grid followed by a drop of 2% phosphotungstic acid solution, pH 7.5. The excess liquid was removed and the grid air-dried.

## RESULTS

### *The Organization of the Gill Epithelium*

The gill epithelium of teleost fish is made of a mosaic of respiratory cells interrupted by chloride cells generally situated at the base of the secondary lamellae (Fig. 1). A few mucous cells are also present.

The major surface of contact between the fish and its outside environment is the layer of long flat respiratory cells. They have polymorphic surface crenellations (microplicae) (Fig. 1*b*), an abundant cell coat, many intracellular vesicles and a prominent Golgi apparatus. The cells are linked by tight junctions and desmosomes, but no gap junctions were observed (Fig. 2*a* and *b*). We examined by freeze fracture many tight junctions between respiratory cells of mullets adapted to fresh or salt water. The depth of these junctions (0.2–0.4 $\mu$m), the number of strands composing them (from 5 to 9), and the pattern of arrangement of the strands do not vary according to whether the fish is adapted to fresh or salt water. The main diffusion barrier between the external environment and the fish gill is the outer plasma membrane of respiratory cells. Freeze-fracture reveals that it has a very peculiar structure (Fig. 3*a* and *b*). Large smooth areas are studded with identical particles (P face) or pits (E face) often arranged as hexagonal arrays (Fig. 3*b*). Upon close examination of the periplasmic face of respiratory cell outer membranes, we noticed much variation in the density of intramembranous particles and in the way they are aggregated. An example of this is given in Fig. 3*b*. It shows the outer membrane P face of two adjacent respiratory cells; in one, particles are scattered at random except in certain areas where clumps have formed, while in the other, almost all particles are aggregated. We saw such large variations not only between neighboring cells but along secondary lamellae, between gills of fish within the same species and among different species. We noticed the same variation in the density and pattern of arrangement of intramembranous particles whether the fishes examined (mullets and eels) lived in fresh or salt water. They were also noted among respiratory cells surrounding the apex of chloride cells in both environments (Figs. 3*c*, 4, and 5*b*).

The fundamental difference between the epithelium of fresh- and salt-water adapted fish concerns the chloride cells. They are situated in cavities or holes opening in the respiratory cell layer (Figs. 1, 3*c*, and 5). In fresh water, each chloride cell is surrounded by respiratory cells linked by tight junctions of the type we describe between respiratory cells (Figs. 3*c* and 4). In contrast, sea water chloride cells form a complex made of two (or more) interdigitating cells linked to each other by shallow junctions thus forming a composite apex (Fig. 6*a* and *b*). This pluri-cellular complex generally forms a deeper apical cavity than that made

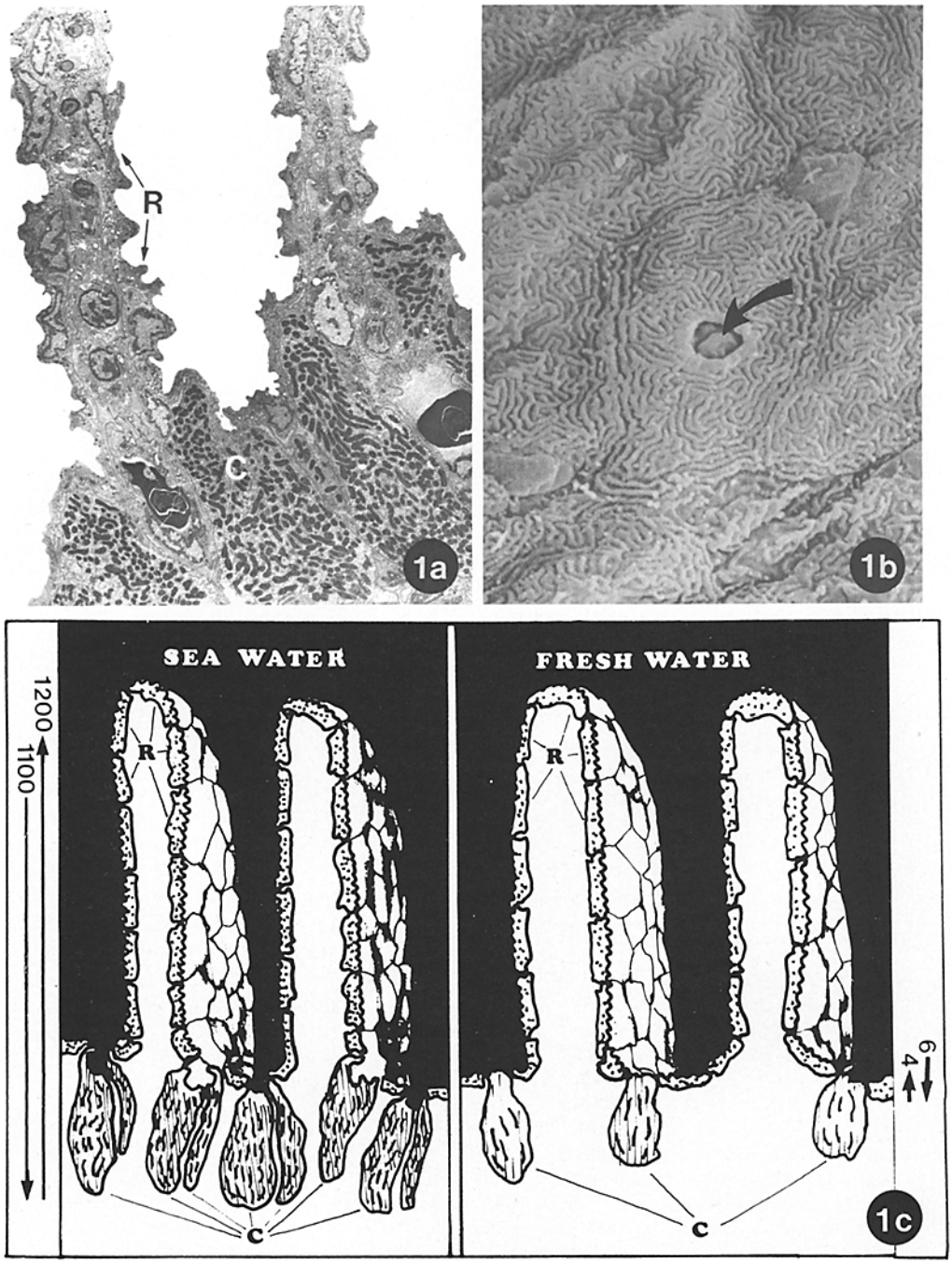


FIGURE 1 General structure of the teleost gill epithelium. (*a*) Seawater mullet. Section through gill filaments. Note chloride cells (*C*) at the base of the filament and respiratory cells (*R*) lining the secondary filaments. × 2,000. (*b*) Seawater eel. Scanning electron microscopy of the surface showing several respiratory cells and the apical cavity of a chloride cell (arrow). × 8,000. (*c*) A perspective view of a section perpendicular to eel gill lamellaes. It shows chloride cells (*C*), their increase and reorganization with seawater adaptation and respiratory cells (*R*). The widely different fluxes of sodium (μmol/h/100 g) through the epithelium are shown by arrows (from reference 25). Fig. 14 shows a detail of the organization of chloride cells in both milieu.

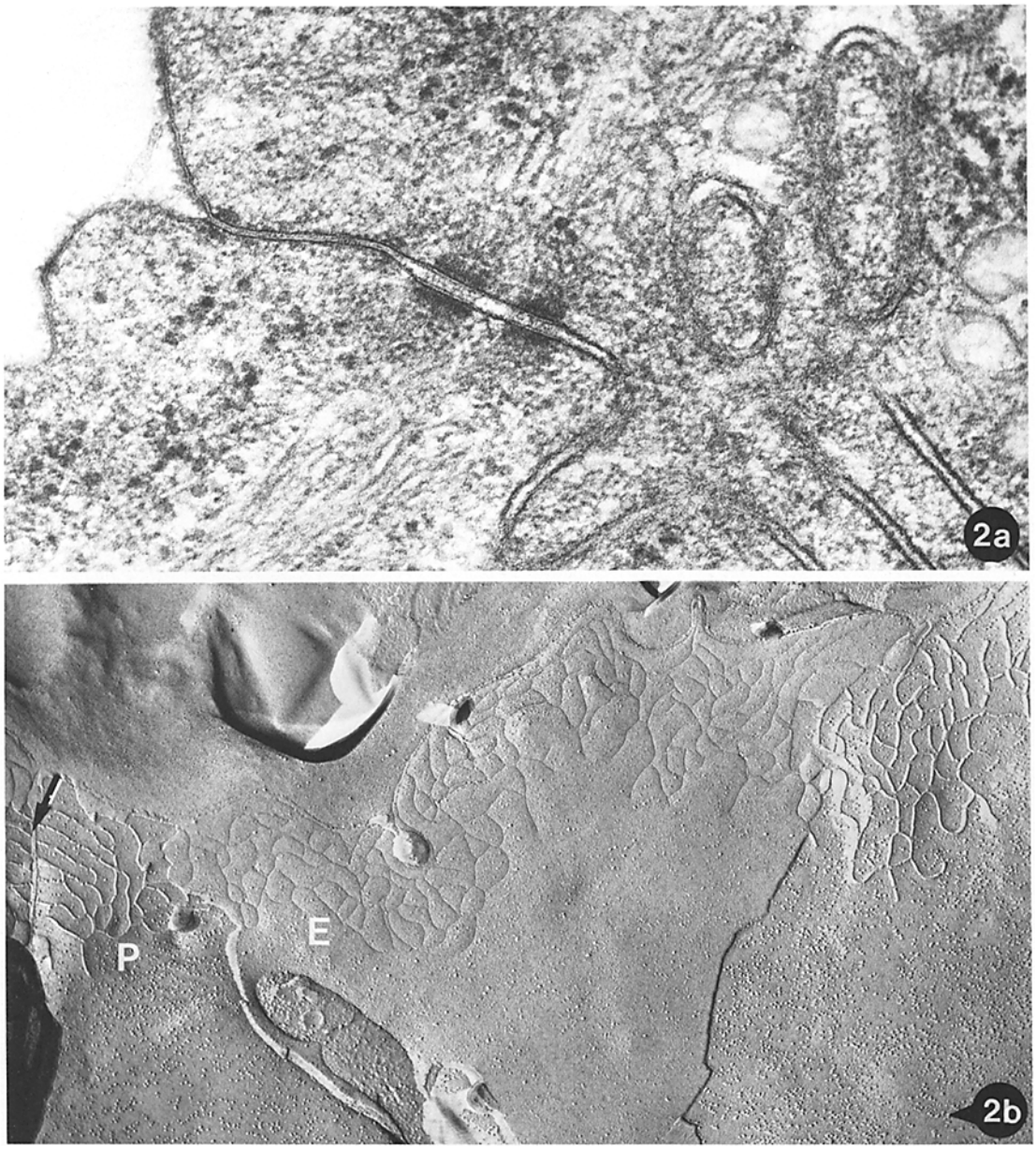


FIGURE 2 Junctions between respiratory cells. (*a*) Freshwater eel. Section through two adjacent respiratory cells showing a long tight junction and two desmosomes. × 70,000. (*b*) Sea water mullet. Fracture through a junction between two respiratory cells. Junctional ridges are on P face and corresponding grooves on the E face; arrow shows edge common to 3 cells. × 44,000. The angle of platinum deposition is indicated by the pointer associated with the figure number.

by the single chloride cell in fresh water. The cells of the complex, like the single chloride cell, are linked to respiratory cells by deep tight junctions (Fig. 6*a*).

### *The Pluricellular Complex of Salt Water Adapted Fish*

In most sections or fractures of the apex of sea-

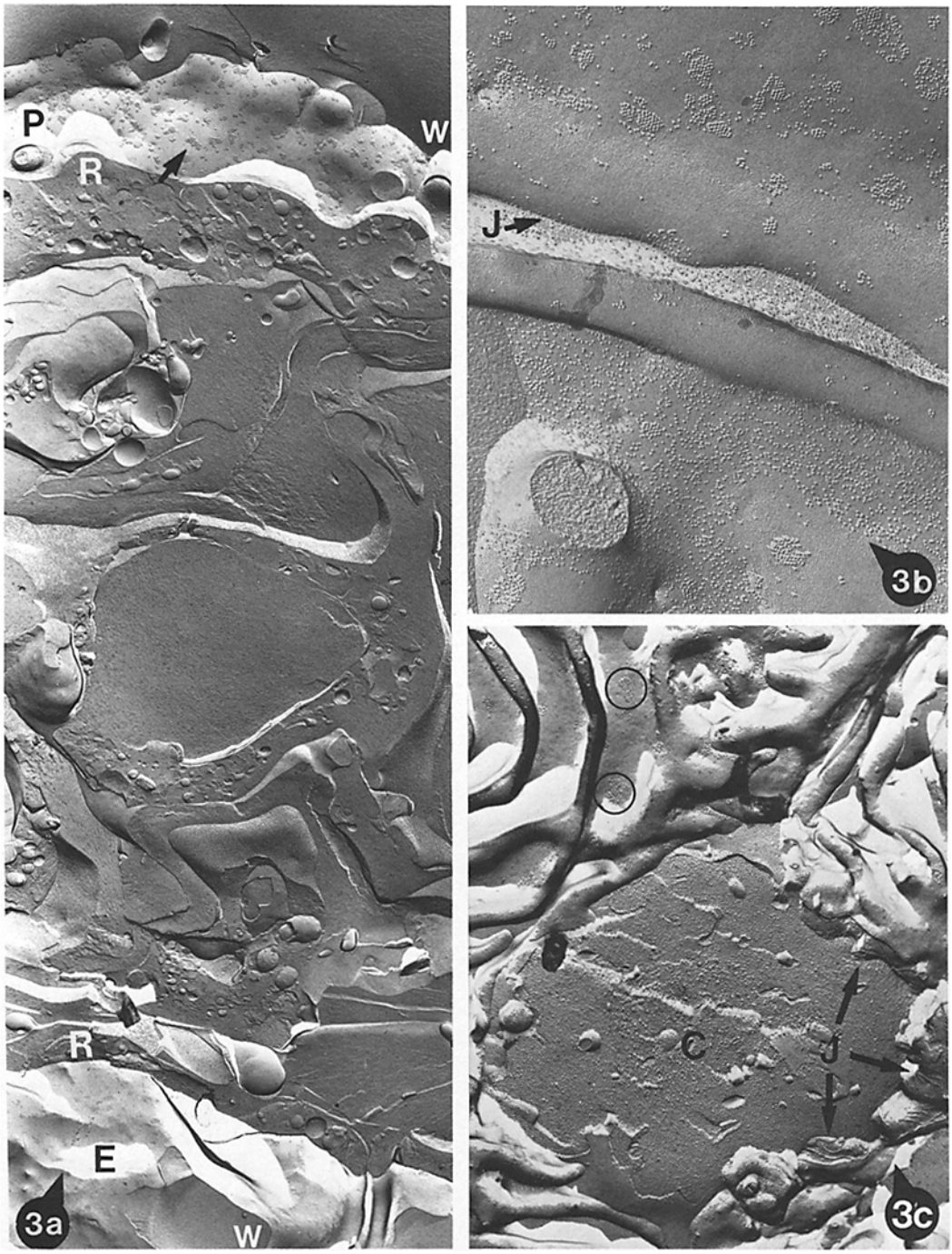


FIGURE 3 The outer plasma membrane of respiratory cells. (*a*) Sea water mullet. Fracture through a gill lamellae, the outer plasma membrane P and E faces of respiratory cells (*R*) are revealed facing the water (*W*). Note the clustered intramembranous particles on the P fracture face (arrow). × 13,000. (*b*) Fresh water mullet. P fracture face of two adjacent cells separated by a tight junction seen face on (*J*). It shows considerable variation in the aggregation of intramembranous particles. × 52,000. (*c*) Sea water mullet. P fracture face of respiratory cells surrounding a chloride cell apical cavity (*C*). Particle aggregates are circled. Note the junction (*J*) between chloride cells and respiratory cells. × 19,000.

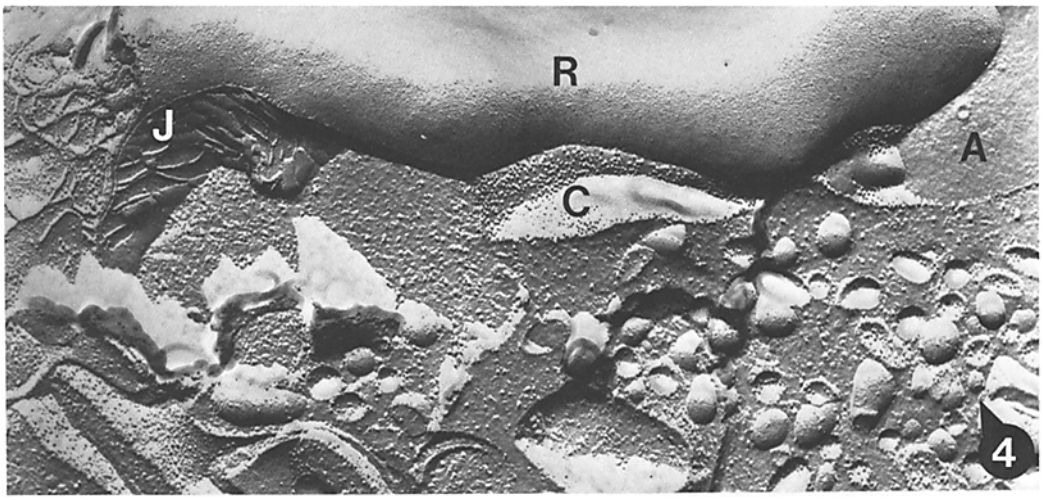


FIGURE 4 Sea water mullet. Tight junction (*J*) between a respiratory cell (*R*) and chloride cell (*C*). Note the striking difference in the density of particles on the P fracture face of the apical membrane of both cell types. *A*: apical cavity. × 47,000.

water chloride cells we observed digitations terminated by shallow junctions, such as those shown in Fig. 6. We never observed such digitations in fresh-water chloride cells but they were extremely numerous in eels and mullets that had been adapted to 200% sea water.

Three kinds of evidence demonstrate that the digitations are arms of sheet-like cells adjacent to chloride cells: (*a*) Glycoproteic material at the apical surface of the cells is demonstrated by colloidal thorium (40) or periodic acid-chromic acid-silver methenamine staining (41). In both cases, the portions of the apical membrane corresponding to the adjacent cell and its interdigitations are more heavily stained than those of the chloride cell (Fig. 7*b*). (*b*) Freeze-fracture provides a way to visualize the apical plasma membrane of chloride cells. In contrast to surrounding respiratory cells, it has a high density of intramembranous particles (see Fig. 4). Interdigitations of adjacent cells are recognized immediately because they have a plasma membrane with a lower density of intramembranous particles than is seen in the chloride cell (Fig. 8*b*). (*c*) Serial sections of the apical region show the continuity of the adjacent cell and the arms found in the apex of the chloride cell (not shown).

### *Structure and Junctions of Interdigitations*

Fig. 8 shows that the tubular reticulum (an extension of the laterobasal plasma membrane) connects with the narrow extracellular space between the arm of the adjacent cell and the chloride cell. In fact, it is common to observe tubes opening in the extracellular space very close to the apical surface (see Figs. 8 and 9*c*). The desmosomal complexes between chloride cells and adjacent cells or their arms (Figs. 8*b* and 9*a*; see also arrows on Fig. 7*a*) stain densely. The desmosomes are connected to thick filaments (~100 Å in diameter) arranged in bundles that show up very clearly when fractured at right angle as in Fig. 8*b*. Fig. 9*a* may provide an explanation of why desmosomes are so noticeable; it shows an adjacent cell sandwiched between a chloride cell and a respiratory cell. The desmosomal fiber bundle links two desmosomes across the adjacent cell. The desmosome and its fiber bundle connecting the chloride cell and the adjacent cell appears more compact than the corresponding structure located between the adjacent cell and the respiratory cell. One gets the same impression looking at the apical junctions between those cells (Fig. 9*a*). The adjacent cell or the chloride cell is separated from the respiratory cell by deep tight junctions. In contrast, the junctions between the chloride cell and the adjacent cell or its arms are very shallow with some dense staining material toward the cytoplasmic side (Fig. 9*a*). They even appear open in some cases (Fig. 9*b*). A fracture through the short junctions gives a clue to their nature, they are one-strand junctions (Fig. 9*c* and *d*) and fundamentally different from all other tight junc-

tions between respiratory cells of the gill tissue which are always constituted of several strands. The use of lanthanum provides another way to differentiate between the deep and shallow junctions. When the seawater fish is examined after 15 min of contact with a lanthanum solution, the heavy metal has penetrated at least part way into the extracellular space of the chloride cell through the short junctions only (Fig. 9*e*). In contrast lanthanum is never seen to penetrate long junctions between other respiratory cells or respiratory cells and chloride cells such as the one seen in Fig. 9*e*. Furthermore, lanthanum does not penetrate into the apex of the chloride cells of the freshwater adapted fish.

### *The Nature of the Adjacent Cell*

The adjacent cell (or cells) is a thin, sheet-like cell whose nucleus and main cytoplasmic mass are situated at the base and on the side of the chloride cells. Only thin digitations reach the apical surface. The cell is filled with vesicles, numerous mitochondria, and a tubular reticulum extension of the laterobasal membrane which is less abundant than that of chloride cells. In contrast, the rough endoplasmic reticulum is more prominent than in typical mature chloride cells. The morphological characteristics of these cells seem to be those of a developing chloride cell.

### *Structure of the Chloride Cell*

This cell has often been described (6, 18, 21, 36). It resembles a pear-shaped bag hanging from the respiratory cell layer (Fig. 1).

The topography of the tubular reticulum and mitochondria which fill the cell is best understood in thick sections colored with the lead technique of Thiery and Rambourg (52) (Figs. 10 and 11). The reticulum is an anastomosing network of tubules in continuity with the basolateral plasma membrane and sheets. The sheets seem to be much more frequent in chloride cells of fish adapted to sea water (Fig. 11*b*).[2] As noticed earlier (17), adaptation to 200% artificial sea water leads to a proliferation of the tubular system and it is not rare to find closely packed tubular arrays. When one looks at them in thin sections (see Fig. 1*a*), chloride cells appear to contain many mitochondria, but it can be seen from Fig. 11*a* that mitochondria must in fact be very few. They are elongated, branched, and wind through the anastomosing network of tubules.

THE TUBULAR RETICULUM: That it is an extension of the laterobasal plasma membrane is clearly seen in thin section or freeze fracture (Fig. 12). We see no particular specialized structure at the place where the plasma membrane invaginates giving rise to a tube. In straight stretches, the size of the tubules is quite constant; ~500 Å in glutaraldehyde fixed thin sections, 500–550 Å when fractured and ~600 Å in negatively-stained preparations. The three techniques reveal that the tubule is made of repeating elements along its length, a factor which may be fundamental in maintaining the constancy of the tubule's diameter (Fig. 12). Freeze fracture gives the best illustration of this; in many preparations irrespective of whether the fish has been adapted to fresh or salt water or of where one looks in the cell, the arrangement of the particles give the P-fracture face of the tubular reticulum a corncob appearance (Fig. 12*b*). Its particles are 70–75 Å in diameter and repeat themselves with a period of 80–100 Å. The E-fracture face is smooth with repeating lines 70–80 Å apart along the tube length. More detailed structure is often suggested as in Fig. 12*c*. The repeating lines seem not to be perpendicular to the axis of the tube. A similar observation is made on negatively stained tubules shown in Fig. 12*d*. In places, we indicate repeating knob-like structures protruding inside and outside the tubule, an observation similar to that made by Dendy et al. (7) on the tubules of pseudobranch chloride cells.

Ritch and Philpott (43) gave the first evidence that the tubular reticulum was made of repeating units; they filled the reticulum with lanthanum by perfusing the gill; tracer staining revealed 14–16 elements disposed 100 Å apart around the tubule. Our observations with freeze fracture agree with this. The tilting of the lines along the tube seen by several techniques suggest that the arrangement of the repeating elements may be helical.

It is known that the reticulum is the site of intense ouabain binding and that microsomal preparations from chloride cells have a high ATPase activity dependent on Na and K and possibly modulated by other ions (18, 19, 27, 45). We believe that we may be visualizing pumping units organized along the reticulum with their ATPase (and Na) site toward the outside and their ouabain

[2] We could make this comparison only on guppies because in mullets, staining of the seawater adapted fish repeatedly colored the mitochondrial network rather than the reticulum.

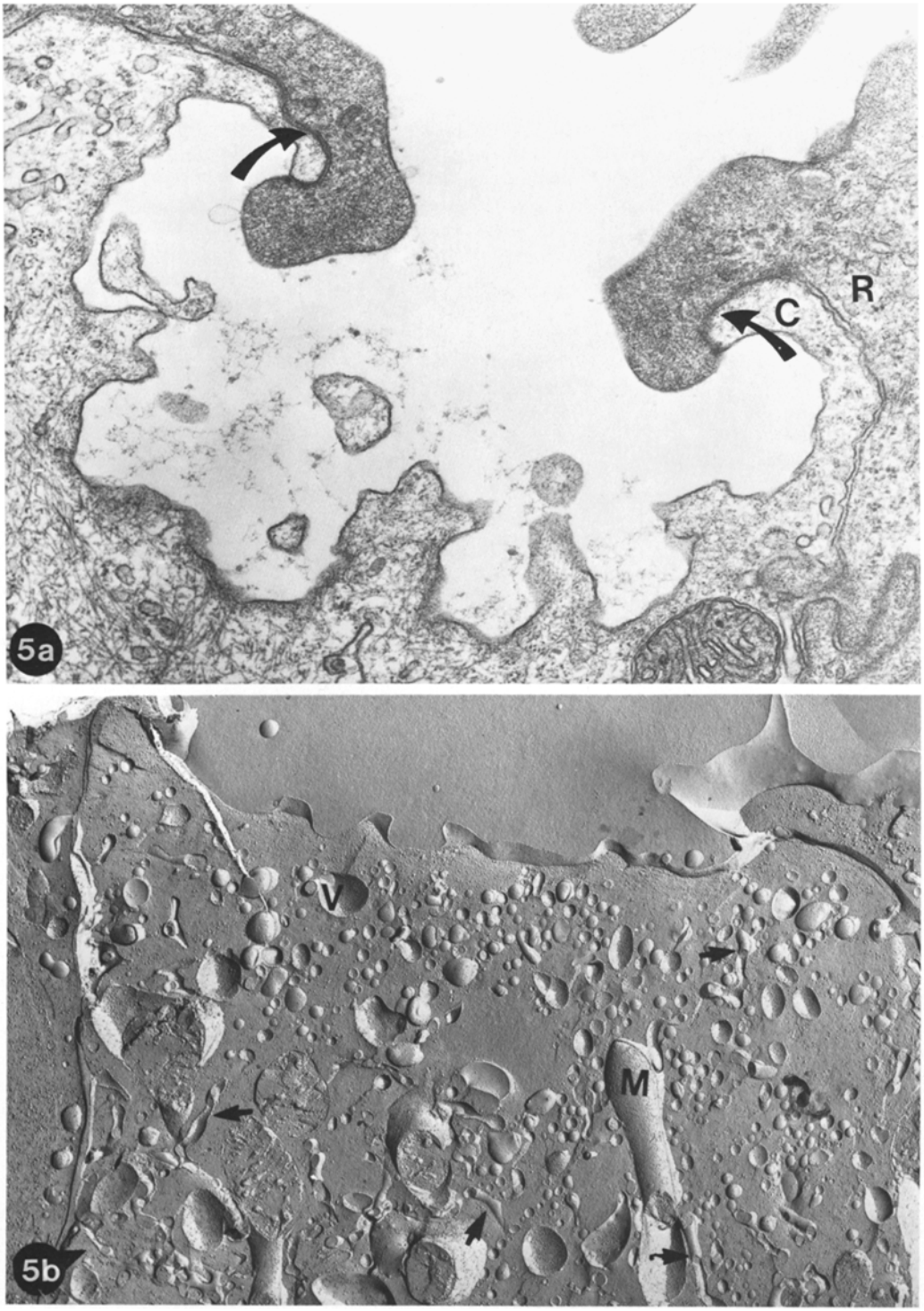


FIGURE 5 Apical cavity of the fresh water chloride cell. (*a*) Fresh water mullet. Section showing the deep tight junctions (arrows) linking chloride (*C*) and respiratory cells (*R*). × 33,000. (*b*) Fresh water mullet. Fracture through the same region. Mitochondria (*M*) vesicles (*V*) and elements of the tubular reticulum (arrows) are shown. × 20,000.

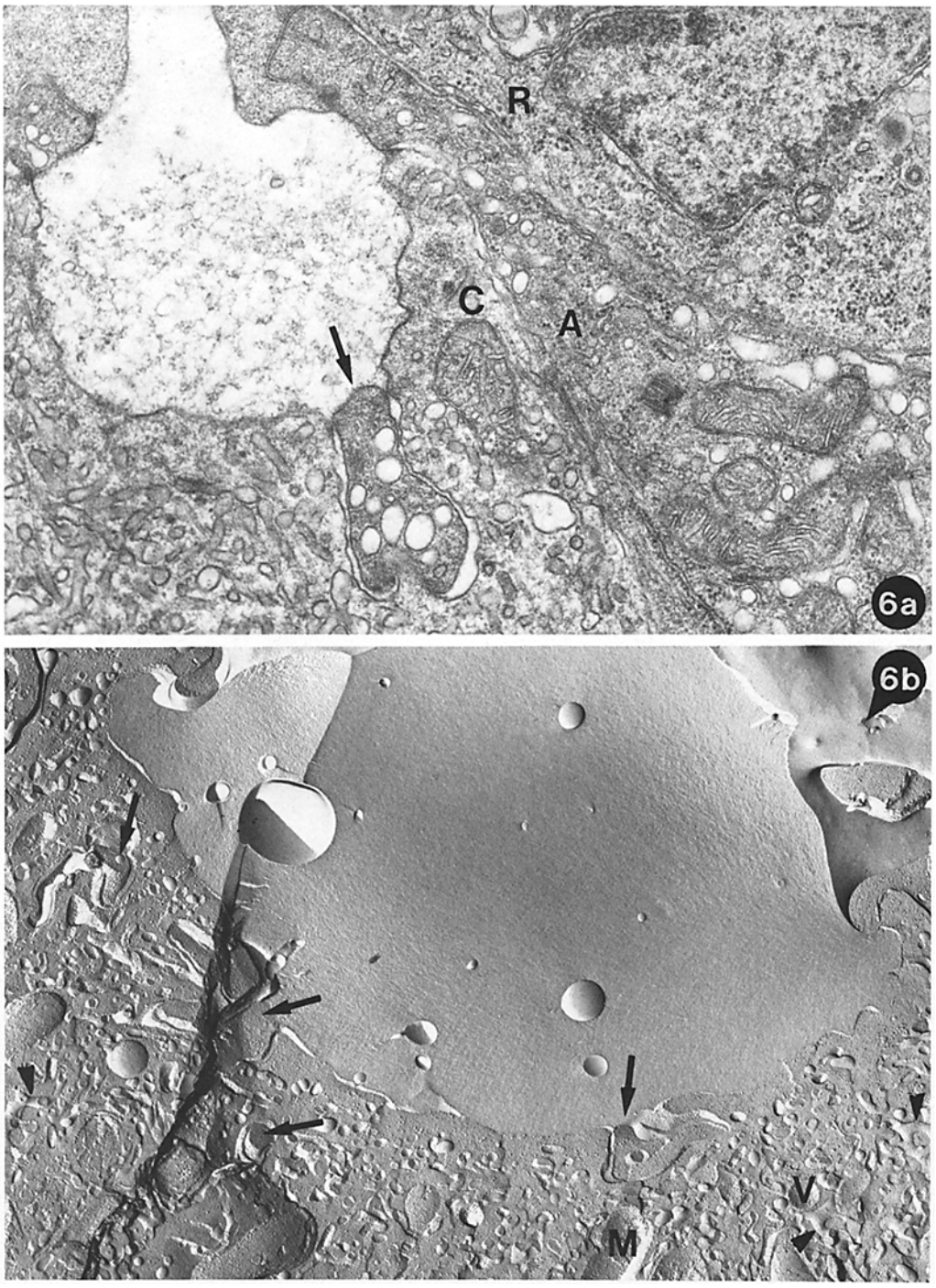


FIGURE 6 Apical cavity of salt water chloride cell. (*a*) Sea water mullet. Section through the cellular complex composing the chloride cell apex. Respiratory cells (*R*) are linked to neighboring cells by tight junctions. The chloride cell (*C*) contains an arm (arrow) of the adjacent cell (*A*). Note shallow junctions between the arm and the chloride cell. The adjacent cell is rich in mitochondria, rough endoplasmic reticulum and tubular reticulum. × 28,000. (*b*) Sea water mullet. Fracture through an apex. Note arms of adjacent cell (long arrows) elements of the tubular reticulum (short arrows) mitochondria (*M*) and vesicles (*V*). × 18,000.

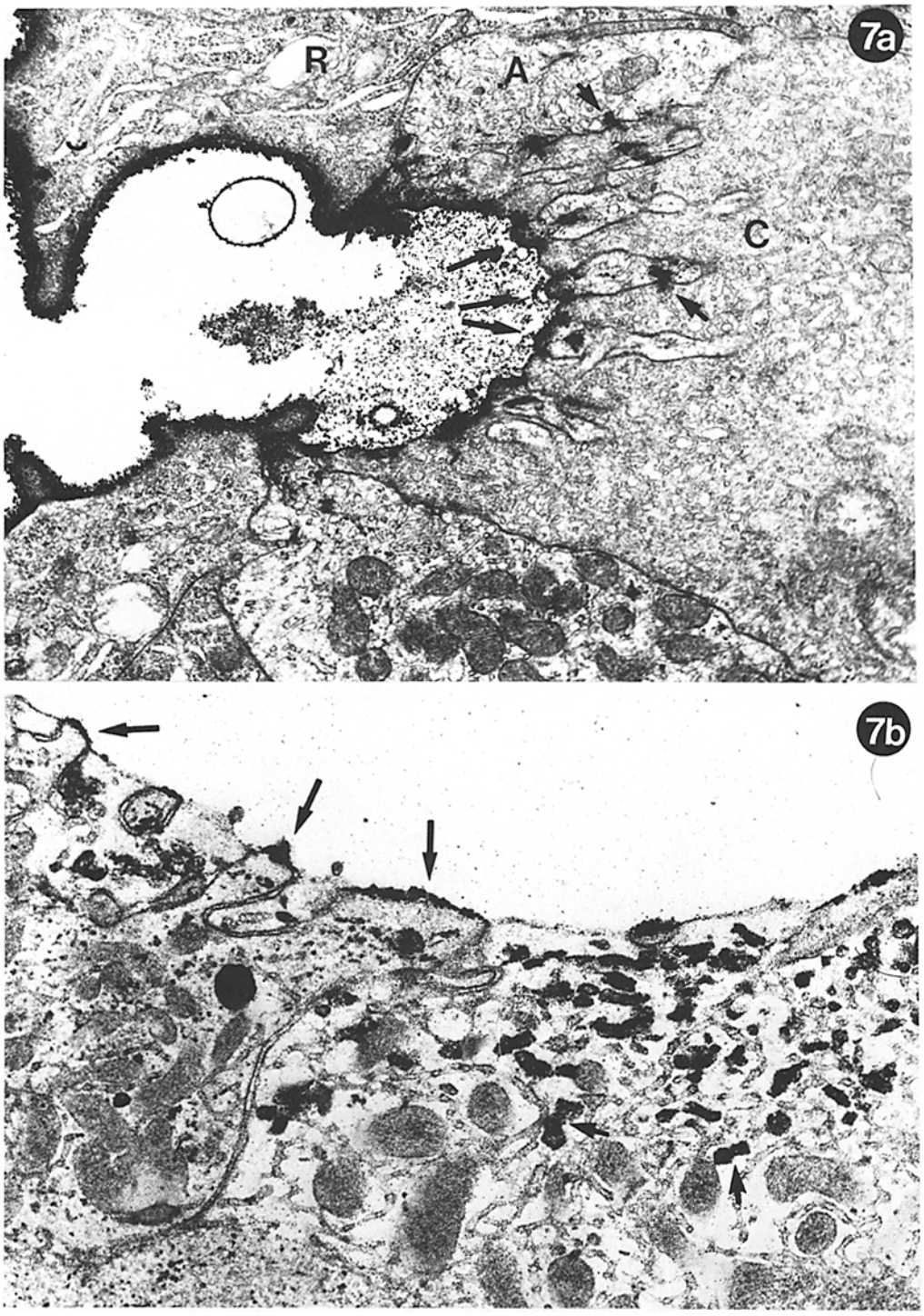


FIGURE 7 Histochemical staining of the apical region of seawater chloride cell. (*a*) Sea water guppy. Colloidal thorium staining reveals the cell coat of respiratory cells (*R*), a typical chloride cell (*C*), and adjacent cell (or cells) (*A*). Note the arms of adjacent cells (long arrows). They are lighter than the chloride cell. The apical membrane staining of the adjacent cell and its arms is thicker than the staining of the chloride cell apical membrane. Note also desmosomes (short arrows). × 14,000. (*b*) Sea water mullet. Periodic acid-chromic-acid-silver methenamine staining of the apical membrane of the chloride cell is weak while the staining of the arms of adjacent cell (long arrows) is very dense. Some apical cytoplasmic material also stains densely (short arrows). × 17,000.

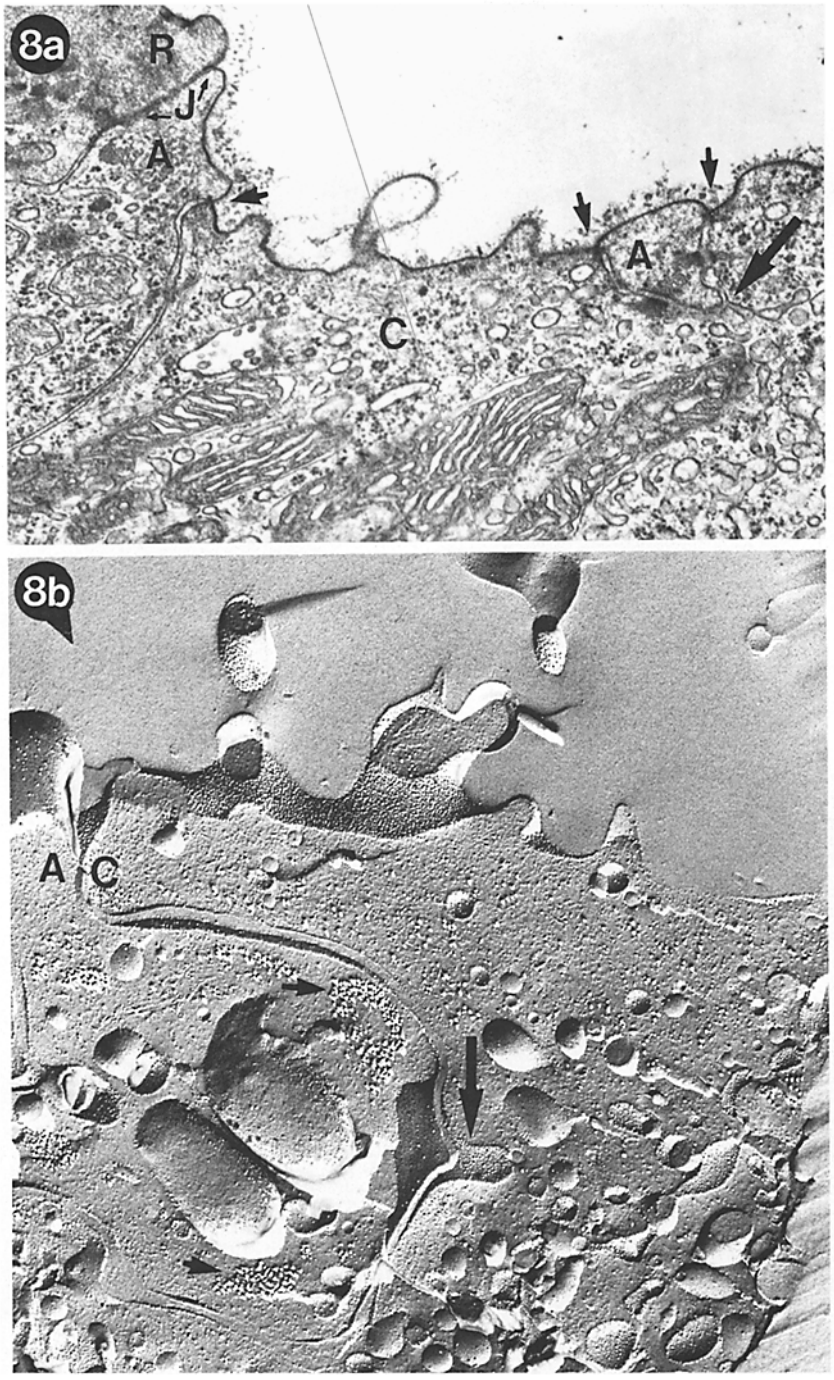


FIGURE 8 Tubular reticulum of chloride cell (*C*) comes close to the surface via the extracellular space created by the arms of the adjacent cell (*A*). (*a*) Sea water eel. Section, note shallow junctions (short arrows) and the tubular reticulum (long arrow), *J* indicates a deep tight junction between an adjacent and a respiratory cell (*R*). (*b*) Sea water eel. Fracture showing the difference in intramembranous particles of the apical plasma membranes of chloride cell (*C*) and adjacent cell arm (*A*). Tubular reticulum (long arrow) and desmosomal fiber bundles (short arrows). × 34,000.

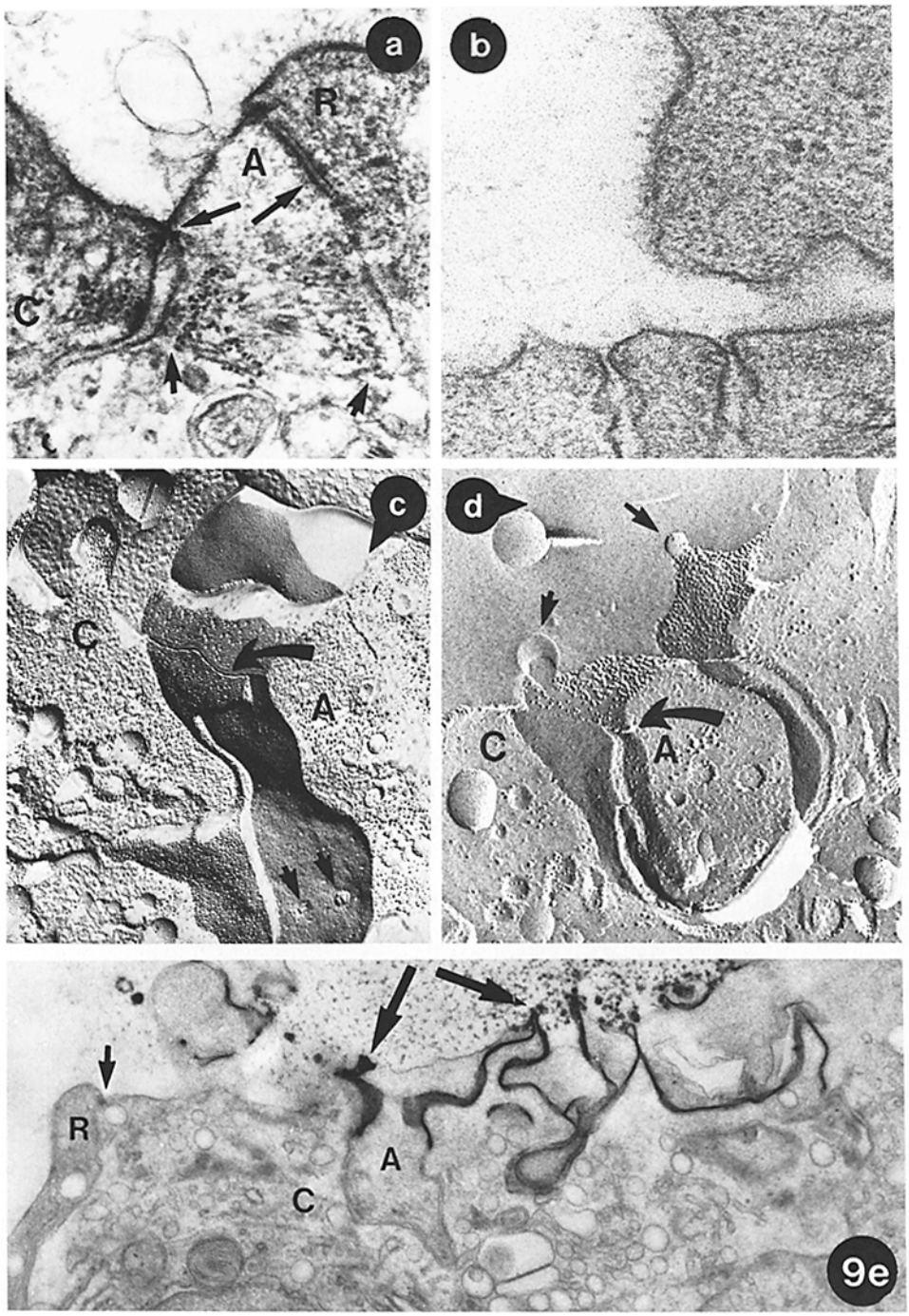


FIGURE 9 Junctions between chloride cell and adjacent cells or thin arms. (*a*) Sea water eel. Junctions (long arrows) and desmosomes (short arrows) between chloride cell (*C*) adjacent cell (*A*) and respiratory cell (*R*). × 69,000. (*b*) Sea water mullet. Junctions between chloride cell and an arm of adjacent cell appear open. × 97,000. (*c* and *d*) Fracture through a chloride cell (*C*) and an adjacent cell arm (*A*) showing that their junctions (arrows) are made of only one strand. In *c*, sea water eel, short arrows show places where the tubular reticulum meets the cell's plasma membrane in communication with the extracellular space very close to the shallow junction. × 35,000. In *d*, sea water mullet, short arrows show blebbing of the apical membranes. × 56,000. (*e*) Sea water guppy. Section of gill treated as described in "experiments with lanthanum." Lanthanum penetrated part way the extracellular space between a chloride cell (*C*) and the arms (*A*) of an adjacent cell (long arrows). The junction between the chloride cell and the respiratory cell (*R*) has not been penetrated (short arrow). × 19,000.

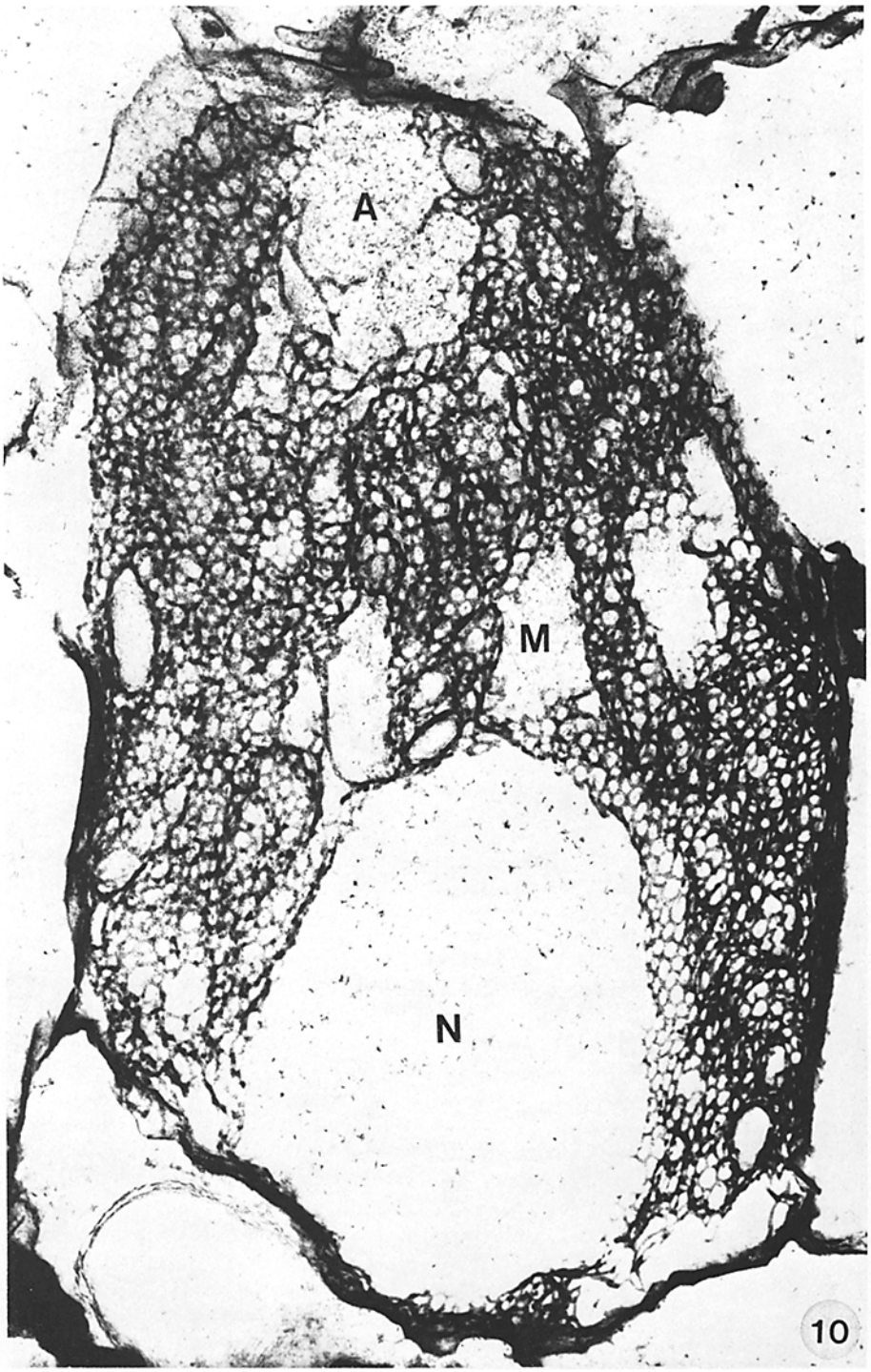


FIGURE 10 Chloride cell tubular reticulum. Freshwater guppy. This is a thick section stained by the lead technique of Thiery and Rambourg (52). The tubular reticulum is stained. Apical cavity and membrane (*A*), Nucleus (*N*) and Mitochondria (*M*) are not stained. × 15,000.

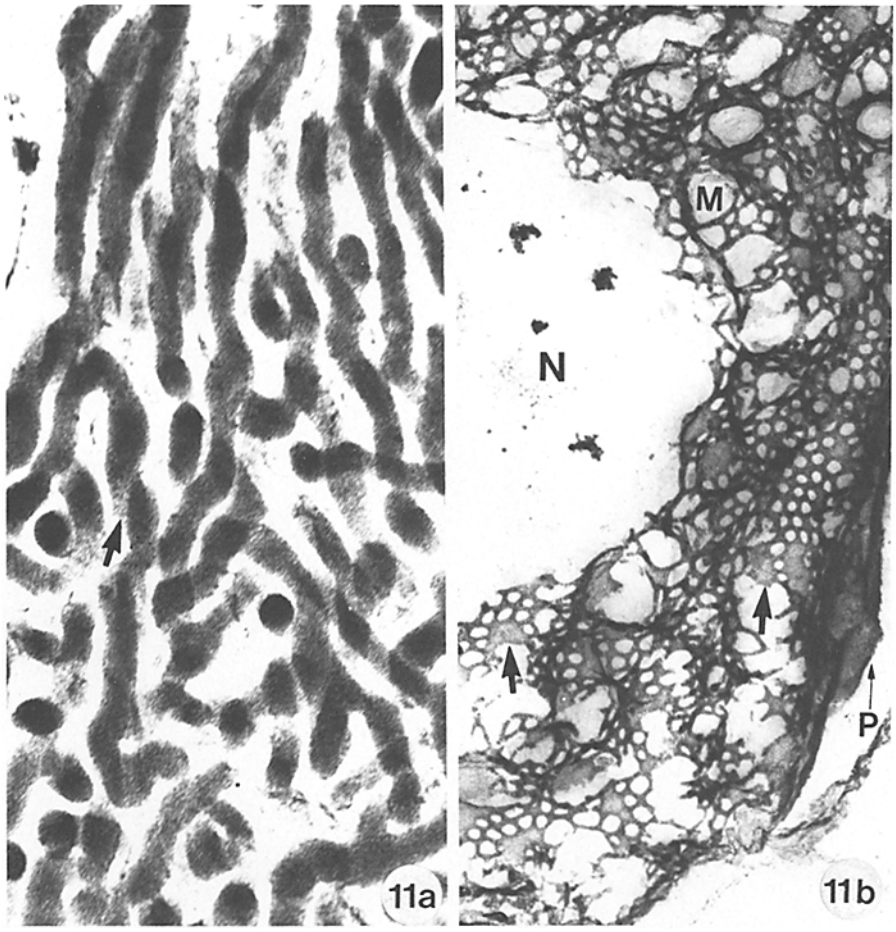


FIGURE 11 (*a*) Salt water mullet. Mitochondrial network revealed by the same staining technique (see footnote 2). It is possible to follow the same mitochondria for great length as it coils upon itself (arrow) × 10,600. (*b*) Salt water guppy: tubular reticulum network. In this case, the same staining procedure as above colors the mitochondria (*M*) very lightly. The tubular network is made of anastomosing tubes interrupted by plaques (arrows). The baso-lateral plasma membrane (*P*) also stains. × 17,000.

(and K) site toward the interior of the tubules. The periodic arrangement of the pumps may confer special functions or may simply be a way to maintain a tube-like structure.

THE APICAL REGION: The mitochondria and tubular reticulum are separated from the apical membrane by an underlying zone particularly rich in microfilaments (Fig. 5*a*). There are numerous "vesicles" in the apical regions that have been interpreted as "secretory vesicles" (36). A close look at many fractured and thin-sectioned apices reveals that they are of two main kinds: (*a*) Coated vesicles (600–850 Å in diameter): these are very numerous in sea water chloride cells but few in fresh water chloride cells (see Fig. 13*a*). (*b*) The so-called "secretory vesicles": these are very difficult to distinguish from sectioned, deep invaginations of the apical plasma membrane and enlargement of the tubular reticulum (see Fig. 13*a*) often filled with a dense staining material. This material (also present within the tubules) was particularly obvious in eels that had been adapted to 200% sea water. It probably is similar to the polyanionic matrix described by Philpott (37). Freeze-fracture images (Fig. 13*a*) and histochemical staining (Fig. 7*b*) suggest that, rather than "vesicles," we may have a vesicular-tubular space between the tubular reticulum or laterobasal membrane and the apical membrane. Neither lanthanum nor peroxidase (put outside or inside)

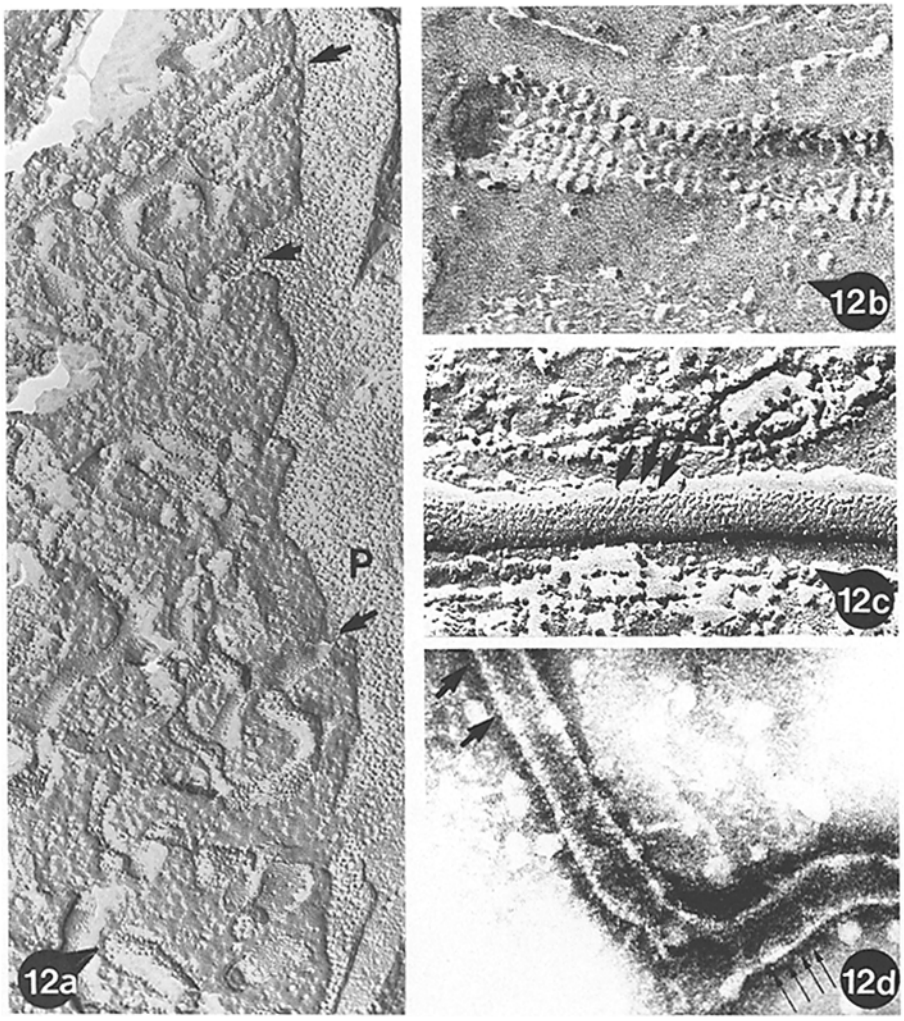


FIGURE 12 (*a*) Sea water eel. Entries of tubules (arrows). The cytoplasmic face (*P*) of the fracture plasma membrane (lateral surface of the chloride cell) and the tubules have identical appearances. × 50,000. (*b*) Sea water eel. P-face of a tubule. Note the corncob-like appearance. × 160,000. (*c*) Sea water mullet. E-face of a tubule. Fine details are suggested by lines repeating themselves along the tubules (arrows). × 140,000. (*d*) Sea water eel. Negative staining of a tubule. Note faint oblique striations (thick arrows) and heads protruding out of tubules (thin arrows). × 110,000.

penetrates this space, but as in the case of the vascular endothelium, elucidation of this point will require careful use of electron dense tracers of various molecular weights (49).

We could see no clear image of pinocytotic or endocytotic process concerning the apical "vesicles." In contrast, we observed consistently a blebbing process of the apical membrane (Figs. 6*b* and 9*d*). This process gives rise to the bubbles found in the apical cavity. Their frequency varies from cell to cell (both in fresh-water or sea-water adapted fish).

## DISCUSSION

### *Salt Water Adaptation Involves a Reorganization of the Epithelium*

Our study points to a fundamental difference in the organization of the gill surface epithelium of fish adapted to salt or fresh water. Salt water

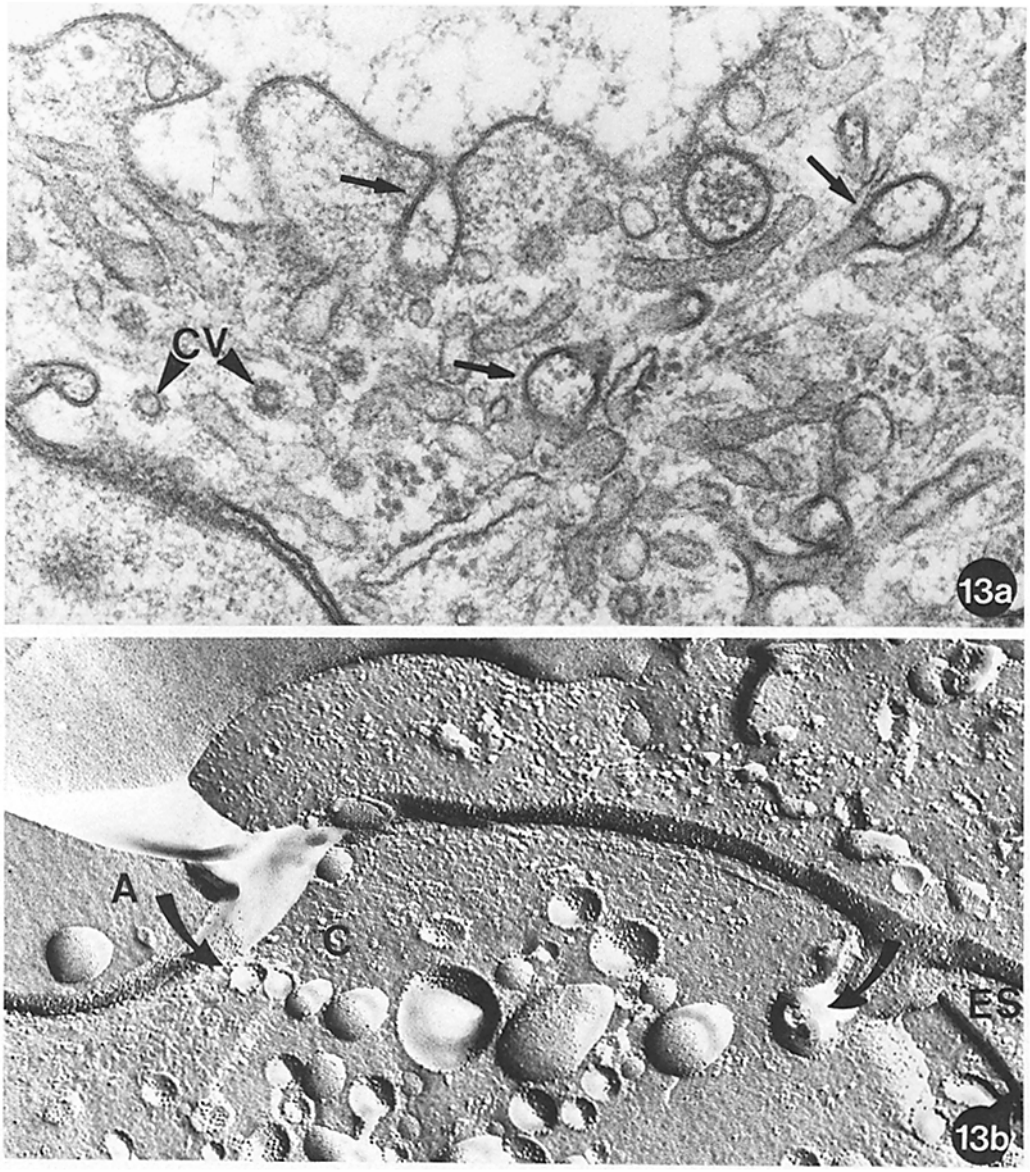


FIGURE 13 (*a*) Sea water mullet. The apical region of the chloride cell shows very abundant coated vesicles (*CV*) and vesicles which seem to be in continuity with the tubular reticulum or the apical plasma membrane (arrows). × 67,000. (*b*) Freshwater mullet. An apical portion of the chloride cell (*C*). A succession of vesicles that could form a tubulovesicular channel between the extracellular space (*ES*) and the external milieu (*A*) is shown by arrows. × 61,000.

adaptation is characterized by the insertion of new cells in the surface epithelium. There are indications that these cells, adjacent to typical chloride cells, are young chloride cells (see above). Preliminary experiments with [$^3$H]thymidine incorporation confirm this hypothesis. The young cells interdigitate with typical chloride cells, creating a composite apex. The salt-water chloride cell is therefore part of a multicellular complex made of several chloride cells, in contrast to the freshwater chloride cell which is generally an isolated cell surrounded by respiratory cells.

Several authors have stressed that the shape of the apical cavity (2, 19, 36) as well as the size of

chloride cells (see references in reference 14) differ in fresh-water and salt-water adapted fish. These observations may be a direct consequence of the development of the multicellular complex we describe. Furthermore, if one closely examines previously published micrographs of chloride cell apices in sea water, it can be seen that arms of interdigitating young cells are present, although unrecognized as such (for example see reference 36, Figs. 5 and 6; reference 48, Fig. 9; and reference 20, Fig. 6).

The multicellular organization characterizing the chloride cells was first described by Dunel and Laurent (9) in the pseudobranch of various salt adaptated teleosts which possess cells closely resembling branchial chloride cells. We also have observed multicellular organization in the opercular epithelium of sea water adapted Fundulus (see also reference 20).

At present we do not know how long it takes for these developmental changes to occur, as our observations only concern long term adaptation to a salt environment (>3 wk). It is known, however, that there is an increased cellular renewal immediately upon transfer of a fish to salt water (6, 34). In this respect it will be interesting to follow the fate of young chloride cells immediately after transfer to salt water and to determine the time it takes for the adjacent cell and its interdigitating arms to reach the apical surface.

Finally, we may suggest that euryhalinity is characterized by the ability to undergo cellular adaptation.

### *Functions of Respiratory Cells and Chloride Cells*

Our study indirectly reinforces the role of the chloride cell as the ionocyte because we show that the major surface of contact between the fish and its external environment, the respiratory cell plasma membrane as well as its tight junctions, are not noticeably different in fishes adapted to a fresh or a salt water environment. Only at the site of the chloride cell is there a major modification of the epithelial layer and the appearance of specialized junctions which will be discussed later.

We would like to draw attention to the respiratory cell's outer plasma membrane, an immense surface (about twice that of the external surface of the fish) (15), which show striking fracture faces. We have recently described these cells in some detail (44) and suggested that the outer plasma membrane must be specialized in a major function related to the remarkable pattern of aggregation of its intramembranous particles. What this function is we do not know yet. It is unlikely to be associated with ionic regulation, although we cannot exclude that mechanisms of acid-base regulation (13), which occur similarly in both fresh- and salt-water adapted fish, take place in those membranes.

### *Junctions between Epithelial Cells*

In both fresh-water and salt-water adapted teleosts, we have shown that the apical tight junctions between the respiratory cells and between respiratory cells and chloride cells are made of numerous interconnected strands. They are similar to those described for tight epithelia known to have a high electrical resistance: amphibian urinary bladder (5, 14), mouse stomach (5, 14), and distal and collecting renal tubules (5, 29, 39). Although some doubts have been raised concerning the relationship between tight junction morphology and electrical resistance of an epithelium (32), we would predict nevertheless that there is little passage of electrolytes between the respiratory cells themselves or between respiratory cells and chloride cells.

Salt water adaptation involves a new type of junction – that in between the chloride cells that form the pluricellular complex. At specific places, such as the apex of chloride cells, shallow junctions are established as a consequence of interdigitations from developing chloride cells. These junctions let lanthanum penetrate while the other epithelial junctions do not. They are typical one-strand junctions resembling those of low resistance epithelia, for example, the thin part of Henlé's loop, or proximal tubules of mammalian or amphibian kidney (3, 5, 16).

We therefore think that salt water adaptation is characterized by the opening of leaky junctions at the site of the chloride cell complex. We do not know exactly how much perimeter these leaky junctions represent in relation to the other junctions of the epithelium. Considering that sections passing just underneath the chloride cell apex reveal many interdigitations, the perimeter of junctions may be quite considerable and may even be proportional to the salinity of the external environment.

Finally, chloride cells are exceptional in that they share deep tight junctions with one neighbor (the respiratory cell), while sharing short, shallow,

tight junctions with the other neighbor (the chloride cell).

### *Transfer of Solutes across the Gill*

As schematized on Fig. 14, there are several possible routes across the epithelium that do not involve the crossing of membranes: (*1*) Passage between the epithelial cells. Based on a comparison of their junctions with those of other epithelia (5, 14), we suggest that this route is fairly impermeable to ions and larger molecules in both fresh-water and salt-water adapted fish. (*2*) The leaky junctions between adjacent chloride cells. This route is characteristic of salt water adaptation, a consequence of the cellular changes we describe. It represents a permanent communication channel from the blood side to the lumen. The communication is via a tubular reticulum made of repeating pumping units supplied in Mg ATP by an enormous mitochondrial space. The key to the cell function is in the understanding of what ions are pumped in and out of the tubes and how they flow toward the external or internal milieu. The presence of leaky junctions would allow ions and possibly small molecules to pass between blood and lumen.[3] (*3*) Tubulovesicular system and coated vesicles. Between the tubular reticulum (which ends short of the apical plasma membrane of the cell) and the membrane there is a vacuole-like space we call a tubulovesicular system. It could be a transient communication channel between the internal and external milieu. We suggest that it resembles what has been described in the vascular endothelium (49). Although there is much difference in its density from cell to cell, it seems to be present in fresh water and sea water and could be responsible for the transit of organic molecules or polysaccharidic materials (24, 30, 31).[4]

Glycoproteins fill the apical cavity and the tubulovesicular space (30). They have also been detected in the tubular reticulum. Their exact function is not known, but Philpott suggested they could be ion-exchangers and participate in ionic regulation (30). Whether they transit via the tubulovesicular space or via coated vesicules remains to be established.

There are good recent discussions by Kyte (23) and Ernst and Mills (12) on how epithelial cells could secrete or absorb salt depending on the leakiness of their apical junctions. It seems to us the concepts they develop would apply well to the gill, at least to explain the movements of sodium. Basically the gill epithelium absorbs chloride and sodium in fresh water from a very dilute environment. Absorption is a result of small in and out fluxes with a small exchange diffusion component (25, 33). In salt water the gill excretes sodium and chloride against large concentration gradients. Excretion is the result of very large in and out fluxes, the major part of which is the exchange diffusion component (see Fig. 1*c*) (25, 33).

In fresh water as in salt water the basolateral pump would build up large concentrations of $Na^+$ in the tubule. In fresh water, Na would enter the apical membrane of the cell and once pumped into the tubular reticulum could only diffuse down its concentration gradient toward the blood side because the apical junction is fairly impermeable to ions (1).

In sea water, Na could penetrate into the cell via laterobasal plasma membranes (NaCl cotransport for example) or apical plasma membrane (possibly due to Na/H or $NH_4$ exchange) (27). In salt water, then, the $Na^+$ pumped into the tubular reticulum can now diffuse toward the blood side or the lumen because the apical junctions are leaky. Since in the seawater fish the blood is electropositive with respect to the external milieu, it could provide the driving force for $Na^+$ secretion via the leaky junctions (26, 35).

Of course, much remains to be done to explain ionic and water movements through the gill epithelium satisfactorily, but we feel our observations provide at least a structural basis for the great ionic permeability of the sea water gill compared with that of the fresh water gill.

### *Salt Excreting Epithelium*

There are other well studied salt secreting epithelia (1). Marine reptiles and birds possess a gland which enables them to excrete the salt absorbed by ingestion from the environment (47). Doyle (8) was amongst the first to study the electron microscopic structure of nasal gland of birds. He suggests that the tubules of the mito-

[3] It has been shown that ouabain outside the fish gill can get into the tubular reticulum of sea water chloride cells (20).

[4] The sea water adapted fish gill is more permeable by a factor of 2 to 3 to small molecules such as inulin and dextran-3,000 than the fresh water adapted fish gill (J. Isaia, personal communication). That increase could correspond to the increase in chloride cell number.

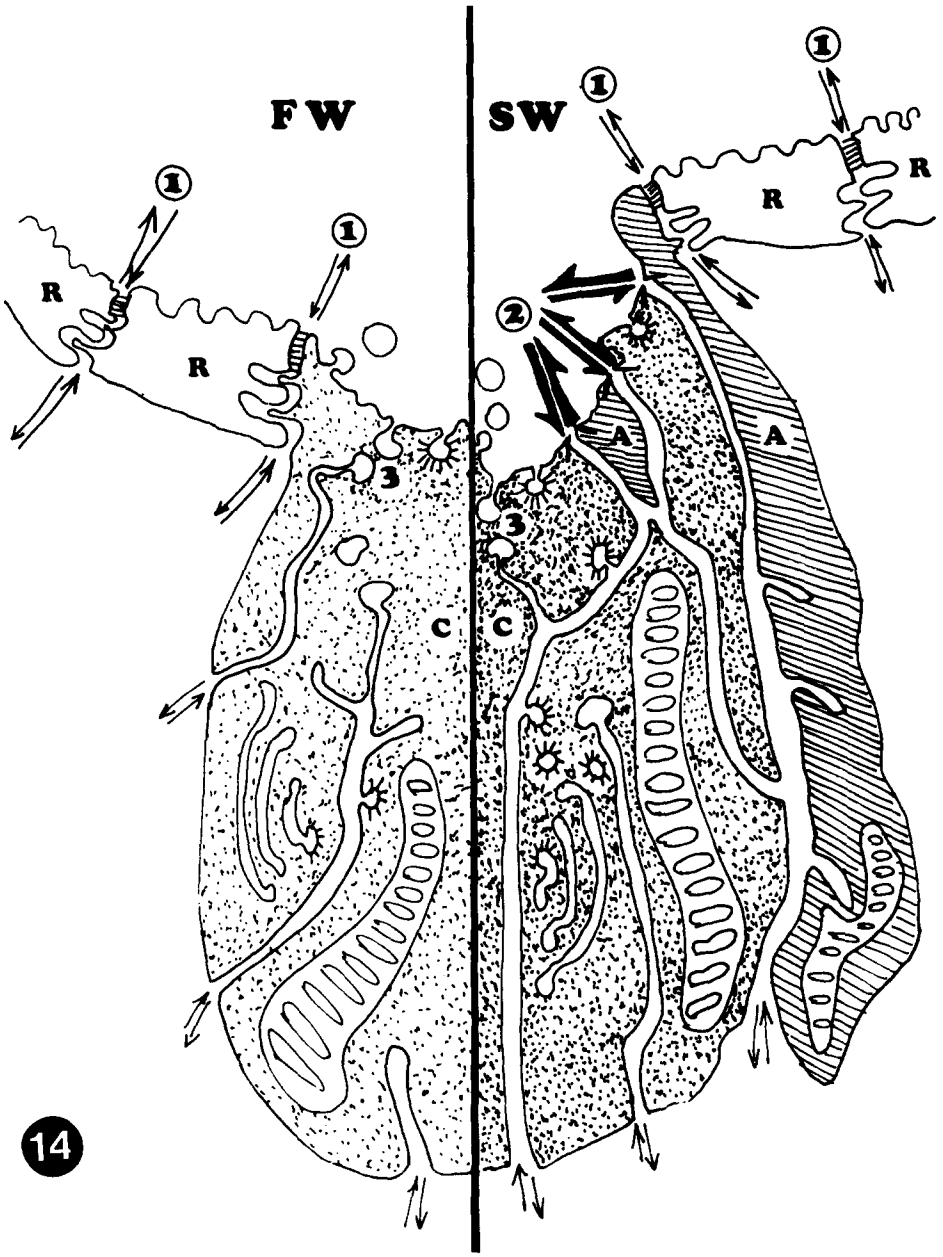


FIGURE 14 This is schematic drawing of the gill epithelium in fresh water (*FW*) or sea water (*SW*) adapted fish. Three possible routes across the epithelium are shown (*1*, *2*, and *3*). In fresh water, only respiratory cells (*R*) and a chloride cell (*C*) are shown linked by tight junctions (*1*). In salt water, the presence of the adjacent cell and its arm (*A*) allows the tubular reticulum and extracellular space to communicate with the external milieu via leaky junctions (*2*). We hypothesize they could also communicate via a tubulo-vesicular system (*3*) present in both sea water and fresh water adapted fish.

chondria rich cells are intercellular spaces resulting from interdigitations of cytoplasmic processes of adjacent cells and that they span the cell from lumen to basal membrane. One illustration (Fig. 6) shows junctions open to the luminal medium. The cell junctions in this tissue have not been examined closely. Leaky junctions have, however, been mentioned to occur in a recent report on the

avian salt gland (10).[5] We suggest that a junctional adaptation of the type described above for the chloride cells also prevails in the salt glands of birds and reptiles.

This suggestion also holds for the rectal gland of the elasmobranchs, where interdigitating processes between neighboring mitochondria-rich cells are obvious (see Fig. 15 in reference 4 and Fig. 2 in reference 53).

In conclusion, amplification of basal membranes, interdigitation of neighboring epithelial cells, as well as the presence of leaky junctions may be common features of salt excreting epithelia.

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## REFERENCES

1. BERRIDGE, M. J., and J. L. OSCHMAN. 1972. Transporting Epithelia. Academic Press, New York.
2. BIERTHER, M. 1970. Die Chloridzellen des Stichlings. *Z. Zellforsch. Mikrosk. Anat.* **107:**421–446.
3. BOULPAEP, E. L. 1971. Electrophysiological properties of the proximal tubule: importance of cellular and intercellular transport pathways. *In* Electrophysiology of Epithelial Cells. Symposia Medica Hoeschst, 1970. G. Giebisch, editor. Schattauer Verlag, Stuttgart.
4. BULGER, R. E. 1963. Fine structure of the rectal (salt-secreting) gland of the spiny dogfish, *Squalus acanthias*. *Anat. Rec.* **147:**95–104.
5. CLAUDE, P., and D. A. GOODENOUGH. 1973. Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. *J. Cell Biol.* **58:**390–400.
6. CONTE, F. P. 1969. Salt Excretion. *In* Fish Physiology, Vol. I W. S. Hoar and D. J. Randall, editors. Academic Press, New York. 241–292.
7. DENDY, L. A., PHILPOTT, C. W., and R. L. DETER. 1973. Localization of $Na^+$, $K^+$-ATPase and other enzymes in teleost pseudobranch. II. Morphological characterization of intact pseudobranch, subcellular fractions, and plasma membrane substructure. *J. Cell Biol.* **57:**689–703.
8. DOYLE, W. L. 1960. The principal cells of the salt-gland of marine birds. *Exp. Cell Res.* **21:**386–393.
9. DUNEL, S., and P. LAURENT. 1973. Ultrastructure comparée de la pseudobranchie chez les téléostéens marins et d'eau douce. *J. Microsc. (Paris).* **16:**53–74.
10. ELLIS, R. A., C. C. GOERTEMILLER JR., and D. L. STETSON. 1977. Significance of extensive "leaky" cell junctions in the avian salt gland. *Nature (Lond.).* **268:**555–556.
11. EPSTEIN, F. H., A. I. KATZ, and G. E. PICKFORD. 1967. Sodium and potassium activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water. *Science (Wash. D. C.).* **156:**1245–1247.
12. ERNST, S. A., and J. W. MILLS. 1977. Basolateral plasma membrane localization of ouabain-sensitive sodium transport sites in the secretory epithelium of the avian salt gland. *J. Cell Biol.* **75:**74–94.
13. EVANS, D. H. 1975. Ionic exchange mechanisms in fish gills. *Comp. Biochem. Physiol. A. Comp. Physiol.* **51:**491–495.
14. FRÖMTER, E., and J. DIAMOND. 1972. Route of passive ion permeation in epithelia. *Nat. New Biol.* **235:**9–13.
15. HUGHES, G. M., and D. E. WRIGHT. 1970. A comparative study of the ultrastructure of the water blood pathway in the secondary lamellae of teleost and elasmobranch fishes. Benthic Forms. *Z. Zellforsch. Mikrosk. Anat.* **104:**478–493.
16. HUMBERT, F., A. GRANDCHAMP, C. PRICAM, A. PERRELET, and L. ORCI. 1976. Morphological changes in tight junctions of *Necturus maculosus* proximal tubules undergoing saline diuresis. *J. Cell Biol.* **69:**90–96.
17. KAMIYA, M., and S. UTIDA. 1968. Changes in activity of Na-K activated adenosine triphosphatase in gills during adaptation of the Japanese eel to sea water. *Comp. Biochem. Physiol.* **26:**675–685.
18. KARNAKY, K. J., JR., S. A. ERNST, and C. W. PHILPOTT. 1976. Teleost chloride cell. I. Response of pupfish *Cyprinodon variegatus* gill Na, K-ATPase, and chloride cell fine structure to various high salinity environments. *J. Cell Biol.* **70:**144–156.
19. KARNAKY, K. J., JR., L. B. KINTER, W. B. KINTER, and C. E. STIRLING. 1976. Teleost chloride cell. II. Autoradiographic localization of gill Na, K-ATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments. *J. Cell Biol.* **70:**157–177.
20. KARNAKY, K. J., JR., and W. B. KINTER. 1977. Killifish opercular skin: a flat epithelium with a high density of chloride cells. *J. Exp. Zool.* **199:**355–364.
21. KEYS, A. B., and E. N. WILLMER. 1932. "Chloride-secreting cells" in the gills of fishes with special reference to the common eel. *J. Physiol. (Lond.).* **76:**368–378.
22. KROGH, A. 1939. "Osmotic Regulation in Aquatic Animals." Cambridge University Press, London and New York.
23. KYTE, J. 1976. Immunoferritin determination of the distribution of $(Na^+ + K^+)$ ATPase over the plasma membranes of renal convoluted tubules. I. Distal segment, and II. Proximal segment. *J. Cell Biol.* **68:**287–318.
24. LAM, T. J. 1968. Evidence of loss of $C^{14}$-inulin via the head region of the threespine stickleback, *Gasterosteus aculeatus*, form *trachurus*. *Comp. Biochem. Physiol.* **28:**459–465.
25. MAETZ, J. 1971. Fish gills: mechanisms of salt transfer in fresh water and sea water. *Philos. Trans. Soc. Lond. Biol. Sci.* **262:**209–251.
26. MAETZ, J. 1974. Origine de la différence de potentiel électrique transbranchiale chez le poisson rouge *Carassius auratus*. Importance de l'ion $Ca^{++}$. *C. R. Hebd. Seances Acad. Sci.* **279:**1277–1280.
27. MAETZ, J., and M. BORNANCIN. 1975. Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. *Forschr. Zool.* **23(2/3):**321–362. Gustav Fisher Verlag Stuttgart.
28. MAETZ, J. 1974. Aspects of adaptation to hypo-osmotic and hyperosmotic environments. *in* Biochemical and biophysical Perspectives in Marine Biology, Vol. 1, D. C. Malins, and J. R. Sargent, editors. Academic Press, New York.
29. MALNIC, G., and G. GIEBISCH. 1972. Some electrical properties of distal tubular epithelium in the rat. *Am. J. Physiol.* **223:**797–808.
30. MASONI, A., and F. GARCIA ROMEU. 1972. Accumulation et excrétion de substances organiques par les cellules à chlorure de la branchie d'*Anguilla anguilla* L. adaptée à l'eau de mer. *Z. Zellforsch. Mikrosk. Anat.* **133:**389–398.
31. MASONI, A., and P. PAYAN. 1974. Urea, inulin and para-aminohippuric acid (PAH) excretion by the gills of the eel, *Anguilla anguilla* L. *Comp. Biochem. Physiol.* **471:**1241–1244.
32. MØLLGÅRD, K., D. H. MALINOWSKA, and N. R. SAUNDERS. 1976. Lack of correlation between tight junction mrophology and permeability properties in developing choroid plexus. *Nature. (Lond.).* **264:**293–294.
33. MOTAIS, R., and F. GARCIA ROMEU. 1972. Transport mechanisms in the teleostean gill and amphibian skin. *Annu. Rev. Physiol.* **34:**141–176.
34. OLIVEREAU, M. 1970. Réaction des cellules à chlorures de la branchie après passage en eau de mer chez l'anguille européenne. *C. R. Seances Soc. Biol. Fil.* **164:**1951–1955.
35. PIC, P., and J. MAETZ. 1975. Différences de potentiel trans-branchial et flux ioniques chez *Mugil capito* adapté à l'eau de mer. Importance de l'ion $Ca^{++}$. *C. R. Hebd. Seances Acad. Sci.* **280:**983–986.
36. PHILPOTT, C. W., and D. E. COPELAND. 1963. Fine structure of chloride cells of three species of *Fundulus*. *J. Cell Biol.* **18:**389–404.
37. PHILPOTT, C. W. 1965. Halide localization in the teleost chloride cell and its identification by selected area electron diffraction. Direct evidence supporting an osmoregulatory function for the sea-water adapted chloride cell of *Fundulus*. *Protoplasma.* **60:**7–23.
38. PISAM, M., and RIPOCHE, P. 1976. Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* **71:**907–920.
39. PRICAM, C., HUMBERT, F., PERRELET, A., and L. ORCI. 1974. A freeze-etch study of the tight junctions of the rat kidney tubules. *Lab. Invest.* **30:**286–291.
40. RAMBOURG, A., and C. P. LEBLOND. 1967. Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. Cell Biol.* **32:**27–53.
41. RAMBOURG, A. 1973. Staining of intracellular glycoproteins. *In* Electron Microscopy and Cytochemistry. E. Wisse, W. Th. Daems, I. Molenaar, and P. Van Duijn, editors. North-Holland Publishing Company, Amsterdam. 245 pp.
42. REVEL, J. P., and M. J. KARNOVSKY. 1967. Hexagonal array of submits in intercellular junctions of the mouse heart and liver. *J. Cell Biol.* **33:**C7–C12.

[5] S. Ernst, personnal communication.

43. RITCH, R., and C. W. PHILPOTT. 1969. Repeating particles associated with an electrolyte-transport membrane. *Exp. Cell Res.* **55**:17–24.
44. SARDET, C. 1977. Ordered arrays of intramembrane particles on the surface of fish gills. *J. Cell Biol. Int. Rep.* **1**:409–418.
45. SARGENT, J. R., and A. J. THOMSON. 1974. The nature and properties of the inducible sodium-plus-potassium ion-dependent adenosine triphosphatase in the gills of eels (*Anguilla anguilla*) adapted to fresh water and sea water. *Biochem. J.* **144**:69–75.
46. SARGENT, J. R., A. J. THOMSON, and M. BORNANCIN. 1975. Activities and localization and succinic dehydrogenase and $Na^+/K^+$-activated adenosine triphosphatase in the gills of fresh water and sea water eels (*Anguilla anguilla*). *Comp. Biochem. Physiol. B. Comp. Biochem.* **51**: 75–79.
47. SCHMIDT-NIELSEN, K. 1965. Physiology of salt glands. *In* Sekretion und Exkretion. Springer-Verlag, Berlin. 269–282.
48. SHIRAI, M., and S. UTIDA. 1970. Development and degeneration of the chloride cell during seawater and freshwater adaptation of the japanese eel *Anguilla anguilla*. *Z. Zellforsch. Mikrosk. Anat.* **103**:247–264.
49. SIMIONESCU, N., and M. SIMIONESCU. 1976. Galloylglucoses of low molecular weight as mordant in electron microscopy. 1. Procedure and evidence for mordanting effect. *J. Cell Biol.* **70**:608–621.
50. SIMIONESCU, N. M. SIMIONESCU, and G. E. PALADE. 1975. Permeability of muscle capillaries to small heme-peptides. Evidence for the existence of patent transendothelial channels. *J. Cell Biol.* **64**:586–607.
51. SMITH, H. W. 1930. The absorption and excretion of water and salts by marine teleosts. *Am. J. Physiol.* **93**:485–505.
52. THIERY, G., and A. RAMBOURG. 1976. A new staining technique for studying thick sections in the electron microscope. *J. Microsc. Biol. Cell.* **26**:103–106.
53. VAN LENNEP, E. W. 1968. Electron microscopic histochemical studies on salt-excreting glands in elasmobranchs and marine catfish. *J. Ultrastruc. Res.* **25**:94–108.