FINE STRUCTURE OF CHLORIDE CELLS FROM THREE SPECIES OF FUNDULUS

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ABSTRACT

A morphological basis for osmoregulation in the teleosts was studied by comparing the fine structure of chloride cells found in epithelia of the gills of three species of fish: *Fundulus heteroclitus* which can survive in a wide range of salinities, and *F. similis* and *F. chrysotus* which are usually restricted to salt water and fresh water environments, respectively. Gills were removed from *F. heteroclitus* which had been laboratory adapted to either sea water or pond water. For a comparison, gills were also removed from the marine *F. similis* and the fresh water *F. chrysotus* which had been adapted to their natural environments. Gill-filaments were fixed in Millonig's phosphate buffered (pH 7.4), 1 per cent osmium tetroxide and were embedded in Epon. Thin sections of filaments were stained with lead hydroxide. The cytoplasm of chloride cells of all three species of *Fundulus* is heavily populated with mitochondria and is filled with tubules of the agranular endoplasmic reticulum (ER). An orderly secretory cycle was indicated for chloride cells of salt water adapted *F. heteroclitus* and the marine *F. similis*. An amorphous material is observed in the agranular ER. Its density increases towards the apical end of the cell. In the apical cytoplasm, tubules of the agranular ER appear to converge and to discharge the amorphous material into an apical cavity. Except for the actual opening of the apical cavity, the distal end of salt water adapted chloride cells is characteristically shielded from the hypertonic environment by thin cytoplasmic flanges projecting from the neighboring epithelial cells. Chloride cells of the fresh water *F. chrysotus* resemble chloride cells of pond water adapted *F. heteroclitus*, in that these cells do not have apical cavities with the functional appearance of those in the sea water adapted forms. The distal end of fresh water adapted chloride cells is typically exposed to the free surface of the gill-filament. The possible function of the cell type is discussed.

INTRODUCTION

The osmotic concentration of fish blood is relatively independent of that of the external medium in both stenohaline and euryhaline teleosts. Since sea water and fresh water environments are, respectively, hypertonic and hypotonic to the bloods of fishes, osmotic work (osmoregulation) is required for the survival of fishes.

Considerable physiological evidence has been presented favoring the osmoregulatory role of the teleost gills in both environmental extremes (15, 17, 35, 36). The gills were found actively to control the osmotic balance by secretion of salt against the gradient of the *milieu exterieur* in marine fishes (15, 16) and absorption of salt against the gradient of the *milieu interieur* in fresh water fishes (17, 18).

In a histological survey of the branchial epithelia of salt water and fresh water fishes, Keys and Willmer (16) identified a cell type which they believed was

responsible for the salt secreting activity. The large, granular, and eosinophilic "chloride secreting cells" were optimally located between the blood and the external medium for their assigned function. These cells, originally described and named by Keys and Willmer (16), will be called "chloride cells," throughout the remainder of this paper, without regard to their possible function(s).

The cytological changes undergone by the chloride cells in response to salt and fresh water adaptation strengthen the hypothesis that these cells are indeed involved in osmoregulation. Liu (19) acclimatized the fresh water paradise fish, *Macropodus opercularis*, to increasing concentrations of salt solution and described a hypertrophy of chloride cells. Copeland (5) sought differences that might occur in chloride cells under more natural conditions by adapting the euryhaline *Fundulus heteroclitus* to varying concentrations of sea water. The sea water adapted fish had characteristic pits or vesicles at the distal end of the cells which disappeared in the fresh water fish. The pits were assumed to be secretory in nature and indicated that the fish was unloading its salt. Copeland placed considerable significance on the observation that fresh water adapted fish could develop the "secretory pit" if they were injected enterically with salt water.

Pettengill and Copeland (29) presented some evidence in favor of the dual role (absorption and excretion) of the chloride cells in *Fundulus* by correlating alkaline phosphatase activity with the energetics required for osmotic work in fresh and salt water; *i.e.*, more alkaline phosphatase was observed in the fresh water adapted condition. This was presumed to be a correlation with the greater total osmotic gradient existing between environment and blood plasma in the fresh water situation. In a later study of the adaptive behavior of chloride cells, Copeland (6) also reported a high density of osmiophilic material, including mitochondria and Golgi elements, in both fresh and sea water adapted cells. Although the chloride cells adapted more rapidly to fresh water than to sea water, Copeland pointed out that the variation in osmiophilic amount and pattern suggested a cyclic behavior in both adaptations.

While the hypothesis of the osmoregulatory role of chloride cells has been contested for various reasons (2, 9, 11, 27, 28, 38), it is perhaps significant that direct evidence against their supposed function is also lacking.

Teleost gill filaments have been studied with the electron microscope in several laboratories, and preliminary observations on the fine structure of chloride cells have been reported by Doyle (8), Kessel and Beams (13, 14), Doyle and Gorecki (9), Straus and Doyle (38), and Philpott (30, 31). From these studies, numerous mitochondria and a well developed agranular endoplasmic reticulum (ER) were found to be characteristic of chloride cells. Doyle and Gorecki (9) have challenged the idea that chloride cells are directly involved in osmoregulation, and Straus and Doyle (38) reported that the "apical pits," which are usually characteristic only of salt water–adapted cells (5, 6, 14, 29–31, 39, 40), were found in guppies adapted to both fresh and salt water.

In view of the extensive histological and histochemical observations by Copeland and his colleagues, *vide supra*, on the chloride cells of the euryhaline fish, *Fundulus heteroclitus*, it is logical to use the same species to review and expand the study of the fine structure of these cells with the electron microscope. Fortunately, two other species of *Fundulus*, *F. chrysotus*, a naturally occurring fresh water fish, and *F. similis*, a naturally occurring marine fish, were available for comparing and relating the fine structure of their chloride cells with the respective fine structures of fresh and sea water adapted *F. heteroclitus*. This paper describes the findings from such a study.

MATERIALS AND METHODS

Adult female *Fundulus heteroclitus* were collected in the vicinity of Woods Hole, Massachusetts, by the Supply Department of the Marine Biological Laboratories during the summer of 1961. Fish were maintained in the laboratory in large stock aquaria provided with running sea water. Presumably the sea water used in this study conforms to Shanklin's (34) analysis of sea water provided to the laboratories of the Marine Biological Laboratory. The temperature range for sea water was 16.8–19°C. Chopped quahog was fed to the fish on alternate days.

The pond water used as an adapting medium for fresh water fish deserves special attention. Copeland (6) studied the adaptive behavior of chloride cells in *Fundulus* and reported that the pits were retained down to at least $\frac{1}{16}$th strength sea water. Subsequent to this study, it has developed that caution should be exercised in adapting fish to tap water with the expectation that chloride cells will show the morphological picture of true fresh water adaptation. Bergeron (personal communication) states that *Fundulus* retains the sea water picture down to $\frac{1}{100}$ concentration. Since chloride cells may retain the morphological picture of sea water adaptation in very dilute solutions and since the hardness and degree of chlorination of tap waters are variable, fish used in this study were adapted to untreated water from Long Pond, the water supply for Woods Hole. An analysis of this water by the Louisiana State Board of Health reported in parts per million showed values for: total hardness (as $CaCO_3$) 10; calcium (as Ca) 2.6; magnesium (as Mg) 0.8; phosphate (as PO_4)

0.05; chlorides (as NaCl) 24.8; chlorides (as Cl) 15.0; and sodium (as Na) by calculation 9.8.

Fresh and salt water adaptations were carried out on experimental animals transferred to plastic pans filled to a depth of 5 cm with three liters of water. Two fish were placed into each pan and adaptations to pond water and sea water were concurrent. Pilot studies confirmed the previous observations of Copeland in 1946 and 1947 (5) that 2 days were adequate for complete morphological adaptation of chloride cells to fresh or salt water. Forty-four hours was the minimum duration of adaptations used in this study. Water was changed frequently in the pans and the experimental fish were not fed in the 24 hours prior to fixation.

Fresh water *Fundulus chrysotus* were seined from ponds below the Mississippi River spillway near Norco, Louisiana, in December, 1961. Water samples taken at the collection site showed that total salinities were less than 0.15 gm/liter. Large adult females were "calmed" overnight in aerated water brought from the collection site, and the gill bars bearing filaments were fixed the next morning.

In November, 1961, marine *Fundulus similis* were seined from isolated salt water pools (above 18.5 gm/liter) located on Grand Isle, Louisiana, in the Gulf of Mexico. The protocol outlined in the preceding paragraph was followed.

The results of this study are based on the electron microscope survey of gill filaments taken from: 14 pond water-adapted *F. heteroclitus*; 16 sea water adapted *F. heteroclitus*; 10 fresh water-adapted (natural environment) *F. chrysotus*; and 10 dilute sea water-adapted (natural environment) *F. similis*. The fish appeared healthy and are believed to be representative.

Christensen and Fawcett (4) and Ito (12) have recently pointed to the labile nature of the agranular reticulum; *i.e.*, the tubular network may coalesce and vesiculate under suboptimal conditions. Various fixatives were tried before the following procedure was found suitably to maintain the integrity of the extensive network of agranular endoplasmic reticulum in the chloride cell.

The fish were sacrificed by brain pithing. Within a 3-minute interval, the second gill arch was removed from the right side of the head and immersed in cold, phosphate buffered (pH 7.4) 1 per cent osmium tetroxide (22). Tissues were kept at 4°C for the 90 minute fixation period and during subsequent tissue handling until dehydration was complete. Fixation was followed by two rapid rinses in distilled water. Ninety-five per cent ethanol was added drop by drop with agitation into the final rinse of distilled water until the fluid volume in the tissue vial was doubled. This solution was replaced at 10 minute intervals by subsequent changes of 50, 70, 85, and 95 per cent ethanol solutions.

The central portion of the gill arch was divided into smaller pieces bearing from two to four filaments, and dehydration was completed in several changes of absolute ethanol. The précis of Luft (20) for Epon 812 infiltration and embedding was followed using equal volumes of mixture "A" and mixture "B." Thin sections were cut on a Porter-Blum microtome and were stained with one of the "lead stains" described by Millonig (23). Tissues were examined with an RCA EMU-3F electron microscope operating at 50 kv.

Gill filaments examined with the light microscope were fixed and embedded as described above, but were sectioned at 1 μ and stained according to Richardson *et al.*, (33).

RESULTS

General

The location and distribution of chloride cells in *Fundulus* essentially follow the pattern described earlier by Copeland (5). In brief, the long columnar cells are found in the stratified epithelium separating the bases of successive lamellae and in the epithelium about the afferent vessel of the gill filament (Fig. 1, inset). The general columnar outline of the chloride cell is frequently interrupted and displaced by adjacent cells of the stratified epithelium. The cells are isolated and may be flanked by other chloride cells (Figs. 1 and 2), epithelial cells, mucous cells, or by any combination of the three cellular types. Since the cells do not always meet the free surface of the gill filament at a constant angle, the thin sections cut for electron microscopy rarely show the full length of the chloride cell.

Mitochondria

Chloride cells of *Fundulus* owe their identity in part to the dense population of mitochondria which are usually uniformly distributed throughout the ground substance (Fig. 4). There may be, however, fewer mitochondria in an apical cortical zone (Fig. 1). A strict baso-apical orientation of mitochondria in respect to the long axis of the cell was not observed.

The mitochondria are usually rod-shaped with an average diameter of 0.4 μ. The average length, as observed by measuring profiles in thin sections is about 1.8 μ with a maximum of 3.0 μ. The rods may be curved and, rarely, split to form Y-shaped profiles.

The organization and fine structure of chloride cell mitochondria conformed to the general de-

scription of these organelles reported by Palade (24). Cristae run more or less transversely across a mitochondrion, but orientations parallel or oblique to the long axis of the organelle are not infrequent (Fig. 4).

Agranular Endoplasmic Reticulum

A characteristic of the chloride cells is the abundance of the agranular endoplasmic reticulum (agranular ER). Most of these "membrane-limited spaces" (32) are disposed as a tubular network with an average lumen of 60 mμ (Figs. 4, 5, and 8). Close relationships, suggesting continuity, are also found between these tubules and the plasma membrane, the flattened cisternae of the granular endoplasmic reticulum, and the clefts of the apical cavities. Networks of the agranular ER are very closely associated with mitochondria, but these do not reach the elaborate and precise orientation described by Copeland and Dalton (7) for the homologous cells in the pseudobranch gland. The distribution of the branching tubules is remarkably constant and quantitative differences from one area of the cytoplasm to the next are slight; exceptions are noted below under descriptions of salt and fresh water adapted chloride cells.

Dense granules or mosaic clumps, stained by lead hydroxide and presumed to be glycogen (PAS-positive in unpublished histochemical studies) are sometimes scattered among the tortuous profiles of the tubules (Fig. 4).

Granular Endoplasmic Reticulum

The granular endoplasmic reticulum (granular ER) (Fig. 7) is not so well represented in the ground substance of chloride cells as is the agranular ER. Elements of the granular ER are, usually, randomly distributed within the cytoplasmic matrix and conform to earlier descriptions of this system (26). Unassociated dense particles that resemble the ribonucleoprotein (RNP) granules are also randomly disposed. Elongate profiles, representing sections through large flattened cisternae, are more often encountered near the nucleus, and continuity from one cisterna to the next by branching can be seen. RNP granules arranged in patterns of spirals, rosettes, and circles are found in the ground substance surrounding the outer surface of these membranes.

The possible continuity of the agranular and granular endoplasmic reticulum has already been mentioned. Additional close associations have been observed between membranes of the granular ER and the outer membrane of the nuclear envelope. These relationships are not unique to the membrane-limited spaces found in chloride cells. They have been observed in many cell types and have been discussed at length by Palade (25) and Porter (32).

Other Cytoplasmic Inclusions and Basement Lamina

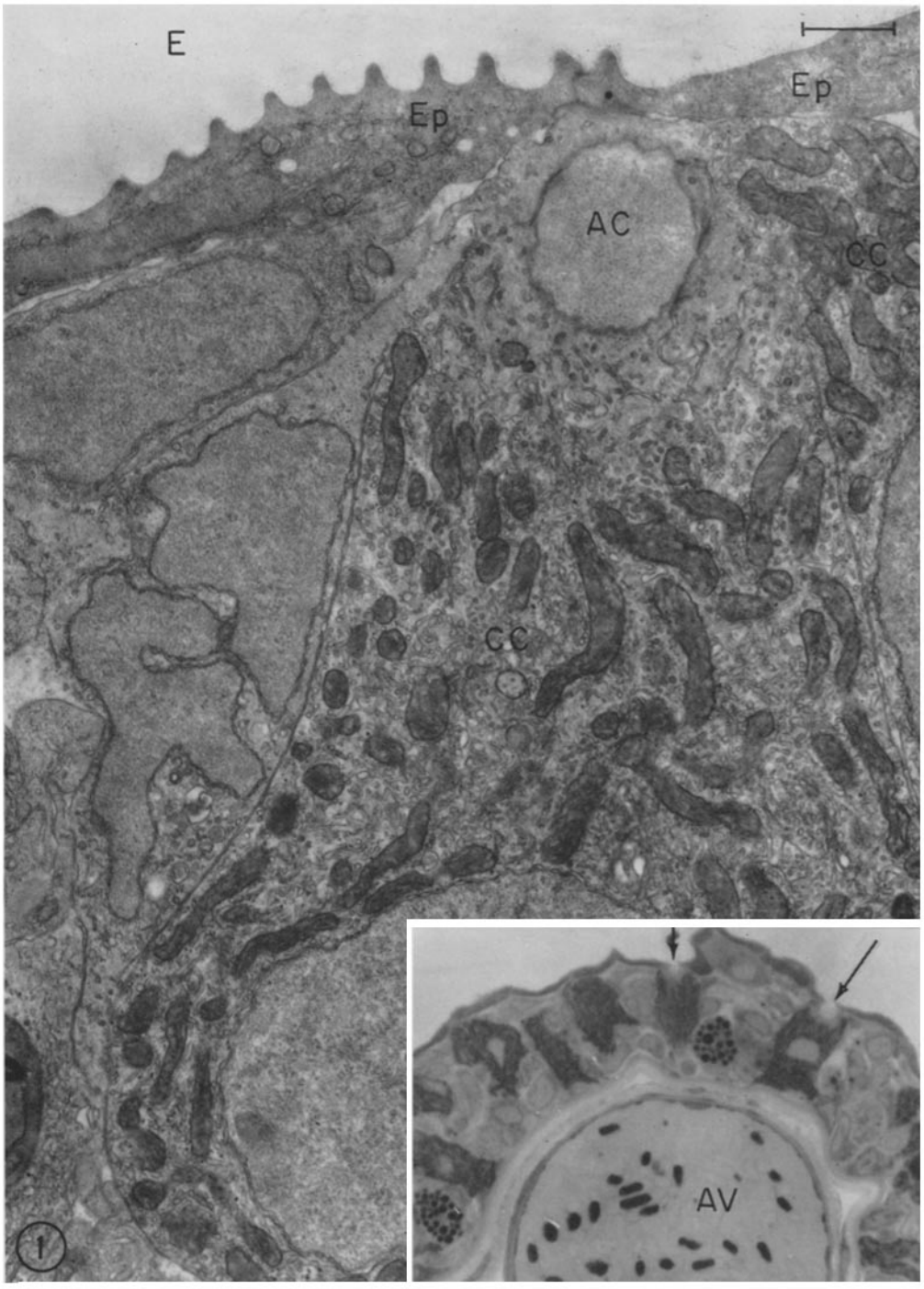
The ground substance of chloride cells is so filled with mitochondria and elements of the endoplasmic reticulum that the Golgi complex is not so conspicuous as in other types of secretory cells. Golgi elements are usually found in a supranuclear position as described by Copeland (6), but certainly the extensive cytoplasmic osmiophilia seen

Key to Abbreviations

AC, apical cavity	*Ep*, epithelial cell
AV, afferent blood vessel	*FB*, fibroblast
BL, basement lamina	*M*, mitochondrion
C, cleft	*N*, nucleus
CC, chloride cell	*RBC*, red blood cell
E, environment	The scale line represents 1 μ.

FIGURE 1 A longitudinal section through a chloride cell of *F. similis*, a fish normally living in sea water. An adjacent chloride cell appears at the right of the picture. Flattened epithelial cells form the free surface of the gill filament and characteristically overlap and cover the apical ends of the chloride cells. × 13,000.

The inset is a light micrograph of a cross-section through the gill filament in the region of the afferent blood vessel. Chloride cells can be seen in longitudinal section extending from the basement lamina toward the free surface of the filament. Arrows point to chloride cell apical cavities which open to the hypertonic environment. × 960.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with the light microscope must now be partially attributed to other cytoplasmic inclusions.

On rare occasions, other inclusions are found in the supranuclear cytoplasm which bear a strong resemblance to the multivesicular bodies described by Sotelo and Porter (37). These bodies in section appear to have a single membrane circumscribing numerous small vesicles embedded in a moderately dense matrix. The average diameter of the bodies is 0.3 μ. The multivesicular-like bodies of chloride cells, however, do not have the centrally disposed nucleoids described for the multivesicular bodies found in the rat ovum (37).

Desmosomes are sometimes seen along the adjoining membranes of the chloride cells and the epithelial cells. Although a detailed study of their fine structure was not attempted, the desmosomes appear as small, dense, parallel plaques made evident by the respective thickenings of the apposing plasma membranes. Ordinarily, tufts of tonofilaments are contributed to these plaques from the cytoplasms of the respective cells (10). Frank bundles of tonofilaments contributing to desmosomes have not been seen within the cytoplasm of chloride cells; however, the cortical ground substance of adjacent epithelial cells contains many conspicuous filamentous and fibrillar forms. It would appear, then, that one of the functions of epithelial cells is to provide a skeletal framework which would help to maintain the architecture of the greater part of the gill epithelium without drawing upon the resources of the more specialized chloride and mucous cell types.

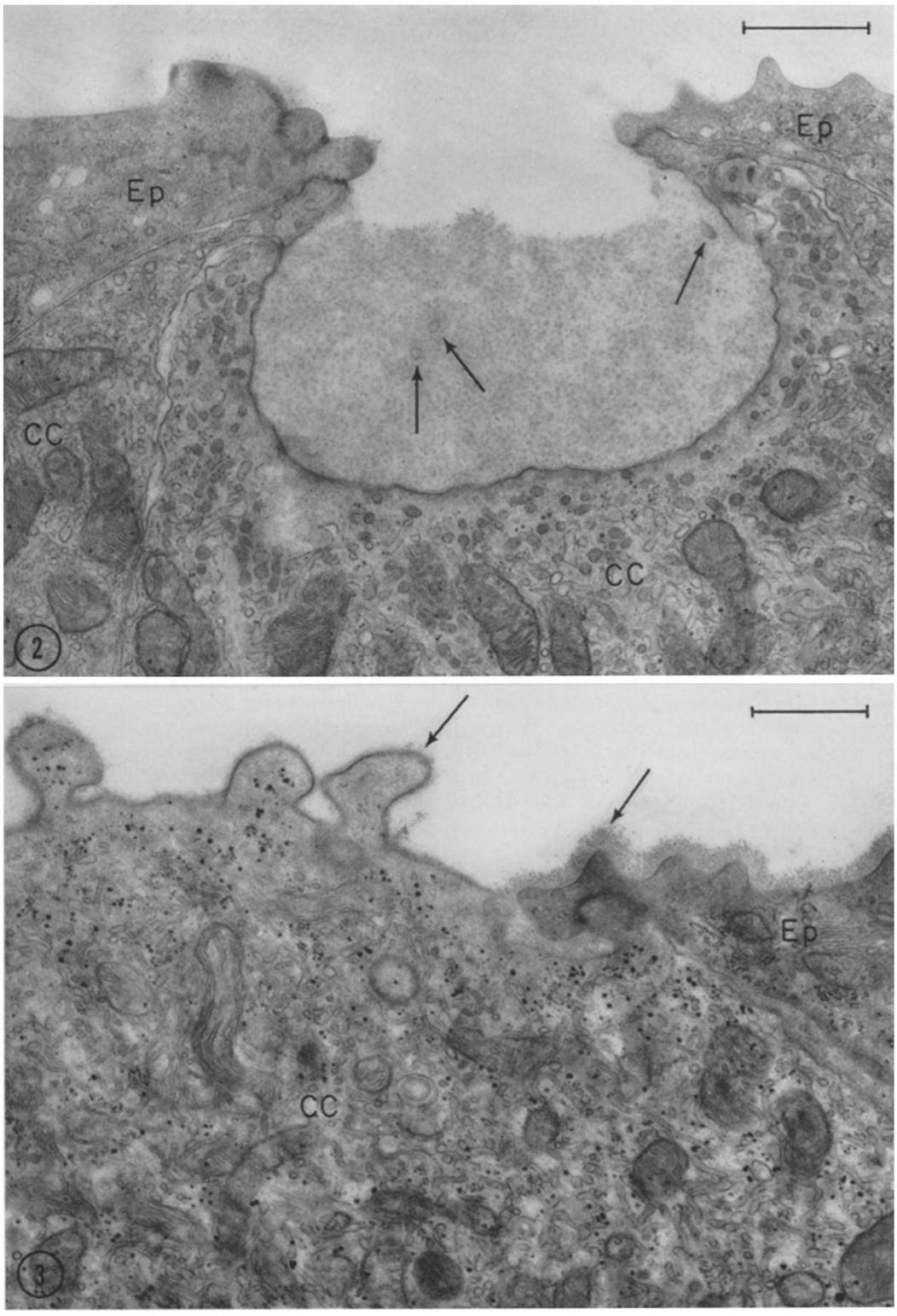
Conspicuous differences in the fine structure of the basal regions of chloride cells were not observed, even when these cells represented different adaptations and different *Fundulus* species. The basement layers reach an approximate thickness of 1.7 μ in the interlamellar regions of the gill filament and 250 mμ at the bases of the lamellae. The thickened, stratified nature of the basement region is shown in Fig. 8, where the plane of section passes perpendicular to the gill epithelium in the region of the afferent blood vessel of the filament. A moderately electron-opaque amorphous basement lamina underlies the chloride cell plasmalemma. Beneath the amorphous layer, several zones of fine fibrils (presumably collagen) lie at right angles to one another. Flattened cells of mesodermal origin are frequently seen in the underlying zones of fine fibrils.

Nucleus

The interphase nucleus of the chloride cells is somewhat irregular in outline and ordinarily has a diameter of the order of 4 μ to 6.8 μ (see Figs. 1 and 4). The nucleus is usually located in the basal part of the cell. The envelope surrounding the nucleus is formed by two membranes separated by a perinuclear space and follows the earlier descriptions of Afzelius, (1); Watson, (41); Porter (32); and Merriam, (21). The outer and inner nuclear membranes sometimes meet to form the circumference of "pores" (diameter 60 mμ) as seen in Fig. 4. Usually an area of increased density can be seen in the "opening" of the "pore" which extends for short distances into both the nucleoplasm and the cytoplasmic matrix. Sections meeting the nuclear envelope obliquely show annuli around the pores. Particulate rings formed from granules which are about the size of RNP particles are also seen in the circumferential margins of the nuclear envelope. The diameters of these rings closely correspond to the diameters of the annuli. Because of the thinness of the sections, it is difficult to ascertain whether the annuli themselves are studded with granules around their margins,

FIGURE 2 Three sea water–adapted chloride cells of *F. heteroclitus* share a single apical cavity which opens to the free surface of the gill filament. In this instance the communicating projections from the two neighboring cells are symmetrically located on each side of the cavity near its opening. Note that the epithelial cells completely cover the chloride cells except for the opening of the cavity itself. The amorphous material within the cavity is in the process of being released. Arrows point toward vesicule-like bodies that are found within the amorphous material of the cavity. × 19,000.

FIGURE 3 The edge of a freely exposed chloride cell in pond water–adapted *F. heteroclitus* is shown. The apical microprojections of the chloride cell (arrow to left) are less uniform than those of the epithelial cell (arrow to right). Notice, also, the difference between the surface coatings of these cells. × 18,000.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or whether the annuli and particulate rings belong to separate structures which are respectively very closely associated with the membranes of the nuclear envelope.

Nucleoli having a size range of 0.5 to 1.1 μ have been observed in all areas of the nucleoplasm of chloride cells. Typically the nucleolus is a more or less compact, irregularly shaped structure which is morphologically heterogeneous. Two zones of the nucleolus can be recognized. The central region is occupied by fine amorphous granules which in turn are surrounded by a peripheral zone of larger particles. Again, the larger particles are similar in size and density to RNP particles. Nucleoli having an irregular fibrillar structure are seldom seen in chloride cells. No more than one nucleolus was observed in a single section of a chloride cell nucleus.

Sea water-adapted Cells of Fundulus heteroclitus and F. similis

The most characteristic and certainly the most widely publicized difference between chloride cells of salt- and fresh water–adapted fish is the vesicle-like cavity (Fig. 1) or crypt (Figs. 2 and 5) found only in the apical cytoplasm of the salt water–adapted cell (5, 6, 14, 29–31, 39, and 40). In former studies of the gill, it was both convenient and descriptive to use the terms "apical vesicle" in reference to the distal vesicle-like cavity of chloride cells and "apical crypt" when the structure was seen to communicate with the external medium. With the electron microscope, it now appears that the "apical vesicle" may be extracellular. The cavity is lined with a membrane that appears identical with that of the free cell surface, and no transition can be detected in those sections where the two are continuous. Although serial sections were not studied, it is possible that the cavity is derived as an invagination which never loses contact with the surface. Hereafter, the term "apical vesicle" will be used with caution and only from the standpoint of this amended view. Obviously, the "apical vesicle" may be indeed an "apical crypt" whose communication with the environment is out of the plane of thin sections.

As a rule, the epithelial cells entirely cap the distal end of the chloride cells except for the actual opening of the apical crypt (Figs. 1, 2, and 5). The sea water–adapted chloride cell, therefore, receives almost no exposure to the hypertonic external medium.

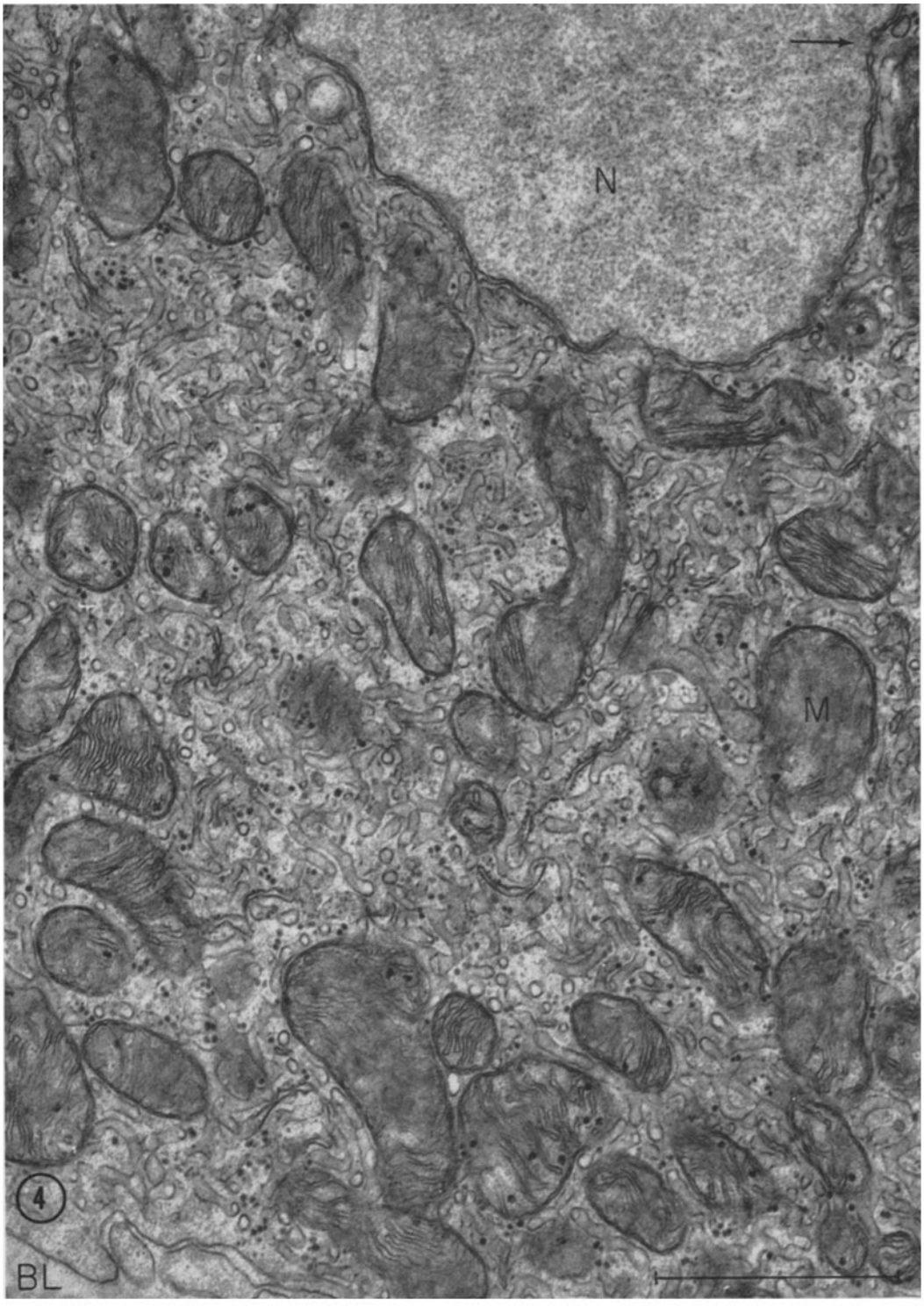
One of the fully developed cavities of the apical cytoplasm *may* have a diameter of 2.75 μ or $\frac{1}{5}$ the diameter of the chloride cell. Numerous clefts arising from the margins of the apical cavities radiate into the surrounding cytoplasm (Figs. 5 and 6). The apposing membranous walls of the clefts appear similar to the membrane lining the apical cavities and hence they are also tentatively interpreted as being identical with the plasmalemma of the chloride cell. A single cell has never been observed to have more than one fully developed cavity. However, it is not unusual for two or three cells to contribute their respective plasma membranes to the margin of a single apical cavity (Fig. 2).

The apical cavities always contain an amorphous material (Figs. 1, 2, 5, and 6). Moderately electron-opaque and ill-defined centers are separated from one another by spaces of about 40 mμ. These centers are surrounded by a less dense cloud of diffuse material. One or two randomly disposed small vesicles may also be seen within the apical cavities (Figs. 2 and 6). Although the diameter of these vesicles corresponds to the diameter of the tubules of the agranular endoplasmic reticulum, their origin is not certain.

In electron micrographs showing the distal portion of the cell, circular profiles with dense contents are frequently encountered in the cytoplasm surrounding the apical cavities (Figs. 1, 2, 5, and 6). Although most of the profiles appear as isolated vesicles, they may represent cross-sections through tubules of the agranular ER, or vacuoles pinched off of the system.

In salt water–adapted cells, the diameter of the

FIGURE 4 The extensive and uniform distribution of the tubular elements of the agranular endoplasmic reticulum can be seen in this longitudinal section through the basal region of the chloride cell. The cell rests on the basement lamina at the lower left. Both transversely and longitudinally oriented cristae occur in the mitochondria. An arrow points toward a "nuclear pore." Widely scattered dense granules, presumably glycogen, are observed in the ground substance. × 39,000.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tubules of the agranular ER increases slightly as this system communicates from the basal to the apical regions of the cytoplasm. There is also an appreciable opacity within the tubules that reaches its greatest density in the terminal vacuoles clustered about the apical cavity.

Histochemical studies (Philpott, unpublished) on salt water–adapted *F. grandis* showed that Alcian blue-positive material (presumably acid mucopolysaccharides) was present in the apical cavities and the surrounding cytoplasm, following a pattern of density similar to the gradient observed in the agranular ER. A further corollary to these histochemical and electron microscope studies on salt water adapted chloride cells is found in Vickers' (39) observation that the rim of the apical "vacuole" in the sea water–adapted guppie was PAS-positive. He has also suggested that an acid mucopolysaccharide was responsible for the positive reaction.

Fresh water-adapted Cells of Fundulus heteroclitus and F. chrysotus

The number and distribution of chloride cells in the gill epithelium appear to be relatively stable regardless of the adaptive state of the fish. Anything resembling apical cavities are rarely encountered in chloride cells of fresh water–adapted *F. heteroclitus* and *F. chrysotus*. Also, in contrast to the sea water condition, these cells usually have exposed distal surfaces. Apical cytoplasmic processes in the form of stubby microprojections are exposed to the environment (Fig. 3). Usually an amorphous material coats the surface of these microprojections as well as the surface of similar, but more uniform, microprojections of epithelial cells. Differences in the amorphous surface coating of these respective cellular types may be seen in Fig. 3, where the coating of the free surface of the epithelial cell is somewhat thicker than the corresponding surface coat on the chloride cell. Although thin cytoplasmic sheets from adjacent epithelial cells may occasionally be seen to overlie a cell, the fresh water adapted cells are typically exposed to the external medium. Except for occasional tubules and vesicles of the agranular ER, the apical region of fresh water adapted chloride cells is relatively free of inclusions. This apical cortical area is somewhat thicker in *F. chrysotus* than in fresh water adapted *F. heteroclitus*.

The membranes of the agranular ER enclose materials of slightly greater electron opacity than the surrounding cytoplasmic matrix, but there is no regional distribution as found within the elements of the agranular ER of sea water chloride cells. Frequency and distribution patterns of mitochondria and elements of the agranular ER are otherwise very much the same throughout the cytoplasm for both fresh– and sea water–adapted cells. This is also true in regard to the elements of the granular ER and unassociated RNP particles. There is no apparent change in the fine structure of either the nucleus or the basal region of the cell which might otherwise be associated with either different adaptations or different *Fundulus* species.

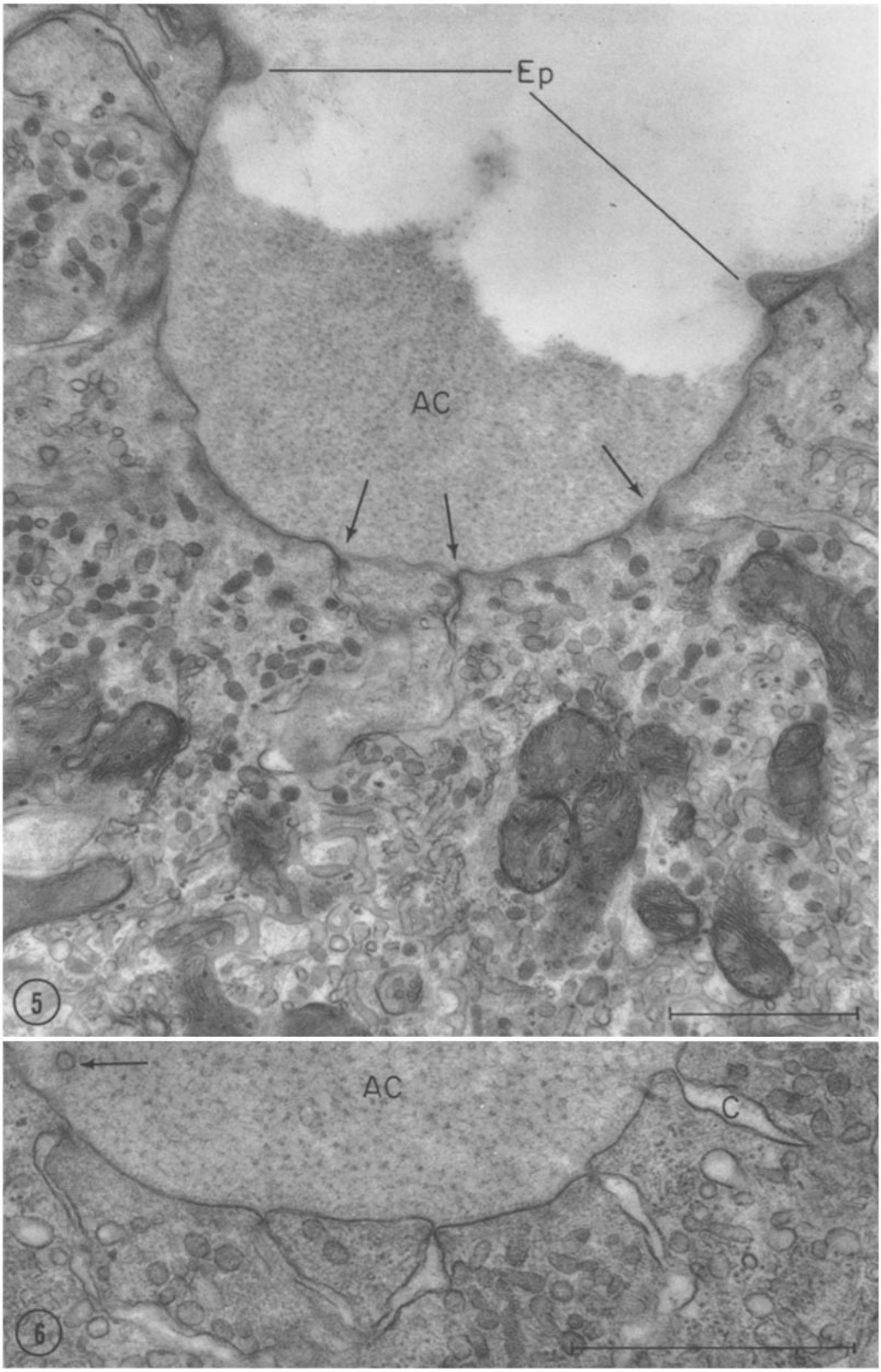
DISCUSSION

In the light microscope studies of the "chloride secretory cells" in the guppy carried out by Vickers (39), a gradation of intermediates between mucous cells and "chloride secretory cells" was reported. In the present investigation, there was no evidence of transformation, both types remaining quite distinct morphologically. As was pointed out by Doyle and Gorecki (9), and supported here, mucous and chloride cells are easily distinguished from one another in the electron microscope.

If osmoregulation is indeed effected by a particular cell type in the gill, then it is only natural that chloride cells should be suspected of having this role. The optimal position between the in-

FIGURE 5 An open apical cavity of a sea water chloride cell of *F. similis*. Clefts radiate from the rim of this apical crypt and can be seen at several points (arrows). The ground substance surrounding the apical cavity contains numerous vesicles (or tubules in cross-section) which may contribute material to the cavity. Notice how cytoplasmic processes from surface epithelial cells extend beyond the rim of the apical cavity. × 29,000.

FIGURE 6 An arrow points toward a vesicule-like body surrounded by the amorphous material within the apical cavity of a sea water chloride cell of *F. similis*. Note the cleft to the right which appears to originate in the apical cytoplasm. × 44,000.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ternal and external environments has been reported in the branchial epithelium and in other highly vascularized areas of the oral, pharyngeal, and opercular epithelium (3). The task of moving ions or molecules against a concentration gradient requires a source of energy for such movement, and the mitochondrion appears to be the principal energy-transfer system of the cell. Of all the cell types which contribute to the branchial epithelium, only the chloride cells are richly endowed with mitochondria, and indeed the remaining cells are puny rivals in this regard. Histochemically, the cell has also been shown to be plentifully supplied with phosphatase enzymes (29).

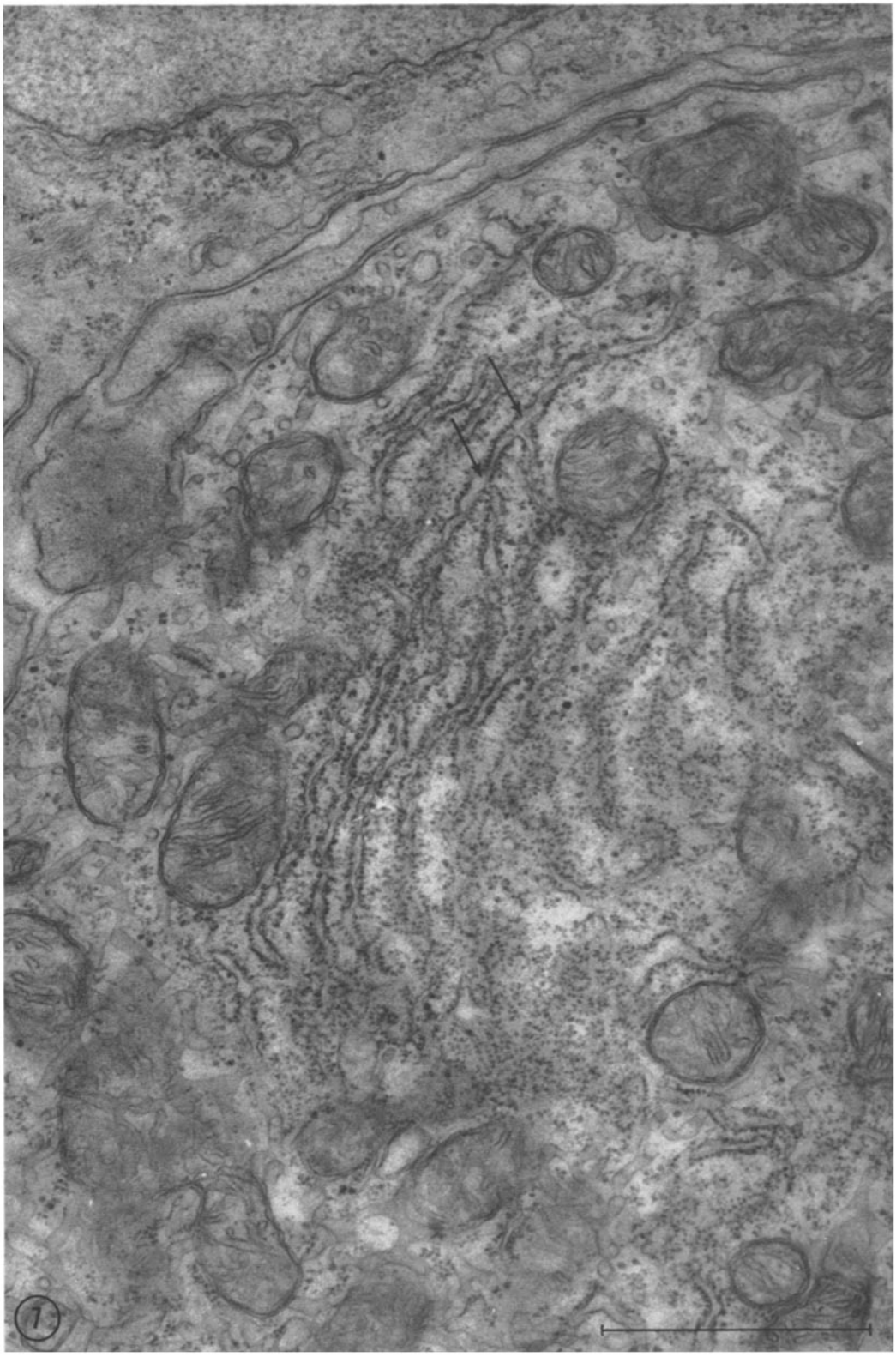
The results of our present study continue to support the possible implication of the chloride cell in salt transportation, in so far as morphological change may indicate functional change. Certainly in *F. heteroclitus*, the apical fine structure of sea water–adapted chloride cells differs strikingly from the apical fine structure of fresh water–adapted cells. Chloride cells of the marine-adapted euryhaline *F. heteroclitus* are strongly homomorphic to chloride cells of *F. similis* which is a normal inhabitant of salt water. Similarly, the chloride cells of *F. heteroclitus* adapted to pond water are comparable with those of *F. chrysotus* which is a fresh water fish by ecological choice.

Let us consider now the sea water–adapted fish, in which the fine structure of the apical cytoplasm of chloride cells is consistent with orderly secretory activity. Tubular elements of the agranular ER become "swollen" as they converge in the region of the apical cavities. An amorphous material found within the enclosed membranes of the agranular ER becomes more dense toward the distal end of the cell, and profiles suggesting interconnections between these membranes and clefts radiating from the apical cavities are common. Apical cavities also contain an amorphous material of an electron opacity similar to that in the adjacent agranular ER. Furthermore, preliminary observations indicate that the contents of both the agranular ER and the apical cavities have histochemical properties of acid mucopolysaccharides. It can be deduced that the relationship between the apical cavities and the agranular ER is more than coincidental. Although the origin of the amorphous material is unknown, one might hypothesize that the membranes of the agranular ER are involved in the concentration and intracellular transport, and the apical cavities in the storage and release, of the substance. If electrolytes are "handled" by the cell, the density gradient would suggest that they are combined with the amorphous material somewhere in the basal area of the cytoplasm and then concentrated during transportation to the apical cavities where release to the environment occurs.

The fact that the sea water–adapted chloride cell is almost entirely separated from the environment by overlying projections of neighboring epithelial cells deserves consideration. It can be argued that the epithelial cells may serve as a protective, impermeable barrier for chloride cells against the hypertonic environment until such time as the loaded apical cavity discharges its contents *via* a narrow opening in the barrier. The barrier is even more complete in the cases where two or three cells may utilize a single cavity. Conversely, the broadly exposed distal surface of the fresh water cell together with the commonly present microvilli suggests an enhancement of total surface area for the purpose of absorbing dilute electrolytes from the surrounding medium, in the sense of Krogh (17, 18).

The pond water–adapted *Fundulus* in this study clearly lacked the apical cavities characteristic of the sea water–adapted animals. Occasionally, surface indentations were seen that might possibly be confused under the light microscope but not under the electron microscope. It must be stated, however, that the transition from the "salt water" picture to the "fresh water" picture occurs at a much lower salinity than ever anticipated. Earlier work (6) indicated the transition to occur at some dilution below $\frac{1}{16}$ sea water. This settled the point of interest at that time, namely that the animal does not go from one histological phase to the other at the iso-osmotic value of its blood. It was

FIGURE 7 Portions of adjacent epithelial cells (upper left) flank a pond water–adapted chloride cell of *F. heteroclitus*. Arrows point toward branching cisternae of the granular endoplasmic reticulum in the chloride cell. RNP granules produce patterns of spirals, rosettes, and circles in the ground substance near the outer surface of the membranous elements of the granular ER. × 41,000.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also concluded that the fish continued to drink the dilute sea water, inducing a continued salt loading. At a later date, one of the authors was disconcerted to find that the tap water in Washington, D.C., would induce the appearance of some apical cavities (unpublished work in A. J. Dalton's laboratory). It is reasonable to interpret this as meaning that the gill secretory mechanism is used so long as *Fundulus* swallows water and so long as that water contains any appreciable "hardness."

This work was supported in part by grant RG-6839 from the National Institutes of Health and grant G-9810 from the National Science Foundation.

Received for publication, November 26, 1962.

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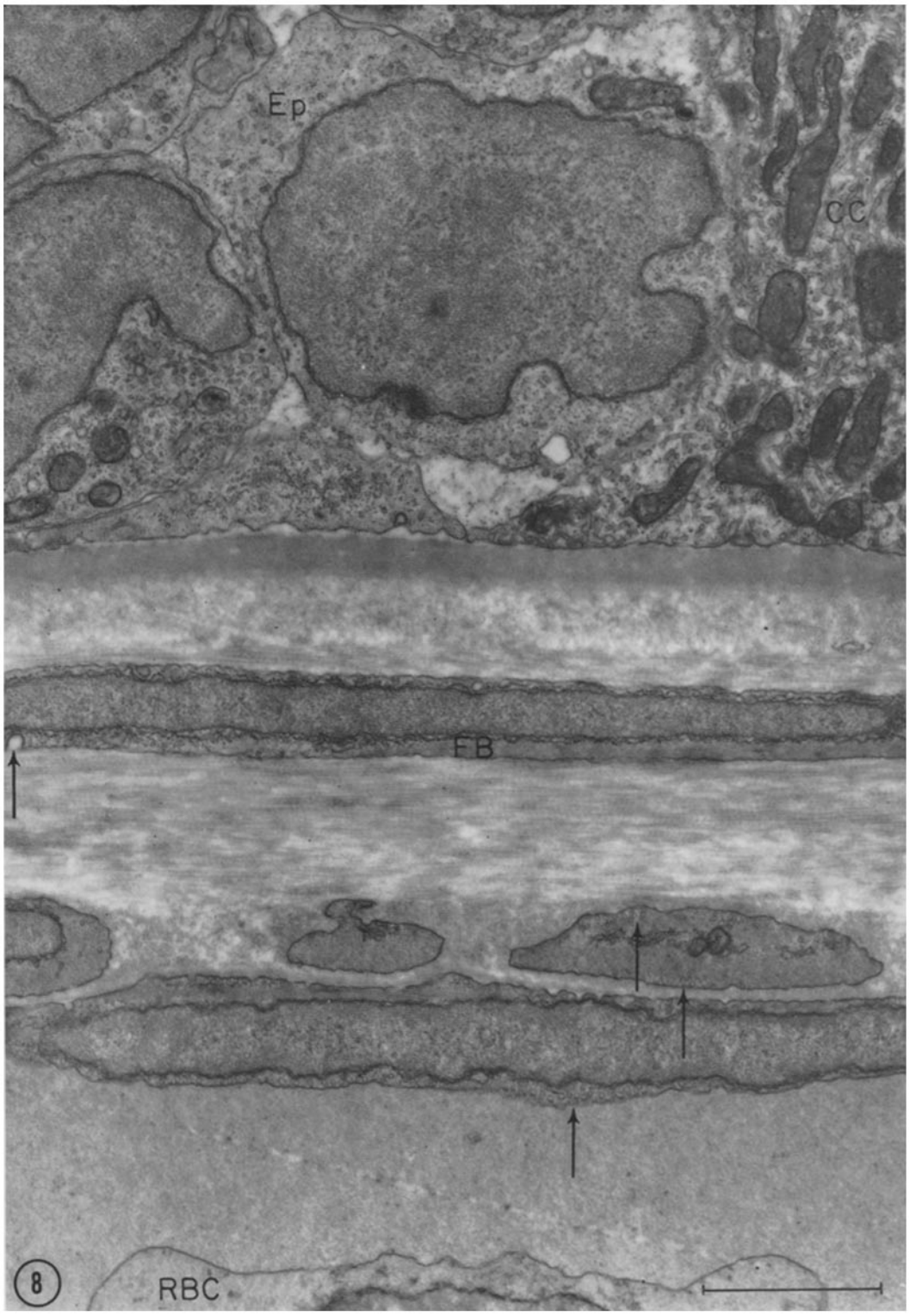
FIGURE 8 A cross-section through the gill filament at the region of the afferent blood vessel. The stratified basement region lies between the chloride cell and the endothelium of the afferent vessel. Arrows point toward possible sites of pinocytotic activity as indicated by numerous invaginations of the plasmalemma and cytoplasmic vesicles of the endothelial cell and fibroblasts. A red blood cell can be seen at bottom center. × 16,500.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