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Structural changes in the zonulae occludentes of the chloride cells of young adult lampreys following acclimation to seawater

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Summary. Thin sections and freeze-fracture replicas have been used to study the structure of the zonulae occludentes of the branchial chloride cells in young adults of the anadromous lamprey *Geotria australis*, caught during their downstream migration to the sea and after acclimation to full-strength seawater (35‰). The chloride cells in the epithelium of the gill filaments of both freshwater- and seawater-acclimated animals form extensive multicellular complexes. In freshwater animals, the majority of chloride cells (64%) are covered by pavement cells and are thus not exposed to the external environment. Most of the other chloride cells are separated from each other by pavement cells or their processes. The zonulae occludentes between chloride cells and pavement cells and between adjacent chloride cells are extensive and characterised by a network of 4 (range 3–7) superimposed strands. In seawater-acclimated animals, the pavement cells cover only 30% of the chloride cells and their processes no longer occur between chloride cells. Whereas the zonulae occludentes between chloride cells and pavement cells are still extensive, those between chloride cells are shallow and comprise only a single strand or two parallel strands. The zonulae occludentes between the chloride cells of lampreys acclimated to seawater are similar to those in the gills of teleosts in seawater, and are thus considered to be leaky and to provide a low-resistance paracellular pathway for the passive transepithelial movement of Na^+.

Key words: Gills – Chloride cells – Tight junctions – Seawater adaptation – Osmoregulatory function – Lamprey, *Geotria australis* (Cyclostomata)

Representatives of the different life-cycle stages of anadromous species of lampreys osmoregulate efficiently in either freshwater or saltwater, primarily through regulating sodium and chloride ions (Galloway 1933; Robertson 1954, 1974; Bull and Morris 1967; Morris 1972, 1980; Beamish 1980; Hardisty et al. 1989). The larval lamprey (ammocoete) is a sedentary, microphagous animal that spends most of its time buried in the soft substrata of streams and rivers (Potter 1980). After a few years, the ammocoete undergoes a radical metamorphosis and, in the case of anadromous species, the resultant young adult enters marine waters where it feeds predominantly on teleost fish (Potter 1980; Youson 1980). Beamish et al. (1978) have shown that the ammocoetes of the anadromous form of *Petromyzon marinus* are unable to osmoregulate in saltwater environments that have an osmotic pressure much greater than that of their blood (ca 225 mOsm kg^{-1}) and that they die in salinities exceeding one third of full-strength seawater (ca 350 mosmol kg^{-1}). In contrast, the fully metamorphosed individuals of anadromous species can be readily acclimated to full-strength seawater (Potter and Huggins 1973; Potter and Beamish 1977; Potter et al. 1980) and can maintain the osmolality of their body fluids at ca 260 mOsm kg^{-1} (Beamish et al. 1978).

The ability of the marine adults of anadromous lampreys to regulate the concentrations of Na^+ und Cl^- in their body fluids at levels far lower than those in their environment parallels the situation in marine teleost fishes (Smith 1930; Parry 1966; Morris 1972; Robertson 1974; Lutz 1975). Furthermore, these adult lampreys possess branchial chloride cells with similar ultrastructural characteristics to those found in teleosts in saltwater (cf. Philpott and Copeland 1963; Peek and Youson 1979a; Karnaky 1986). Details of the way in which this cell type is involved in regulating Na^+ and Cl^- in marine teleosts is based mainly on work carried out on euryhaline species, such as the killifish (*Fundulus heteroclitus*) and tilapia (*Oreochromis mossambicus*). Whereas the secretion of chloride by this cell type occurs actively via a transcellular route, sodium transport is passive and paracellular (Karnaky et al. 1976a, b; Degnan et al. 1977; Ernst et al. 1980; Karnaky 1980, 1986; Foskett and Scheffey 1982; Eriksson et al. 1985; Foskett and Machen 1985). The passive transport of Na^+ across

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the gill epithelium is facilitated by the presence of shallow zonulae occludentes between the chloride cells, which are arranged in multicellular units (Philpott and Copeland 1963; Sardet et al. 1979; Ernst et al. 1980; Hwang and Hirano 1985; Hwang 1987; Pisam et al. 1988, 1990; King et al. 1989).

Although freeze-fracture studies have been used to elucidate the structural significance of the occluding junctions in the osmoregulatory epithelia of teleosts, elasmobranchs and birds in saltwater (Ellis et al. 1977; Riddle and Ernst 1979; Sardet et al. 1979; Ernst et al. 1980, 1981), there have been no comparable investigations of the gills of lampreys. The present study on lampreys was therefore undertaken to determine whether the chloride cells of this agnathan group have the same structural prerequisites for the passive, paracellular movement of sodium as those in teleost fish. Emphasis was also placed on describing the changes in the relationships that occur between chloride cells when the lamprey passes from freshwater to seawater.

Materials and methods

Fully metamorphosed *Geotria australis* were caught during their downstream migration to the sea in the Donnelly River in south-western Australia. They were held in laboratory aquaria containing well-aerated freshwater. Twelve of these lampreys were acclimated to full-strength seawater (35‰) by transferring them for 4 days firstly to 1/3 seawater and then to 2/3 seawater. After 1 week in full-strength seawater, these animals were killed following anaesthesia in a solution of 0.01% benzocaine. Twelve young adults that had been held in freshwater were sacrificed at the same time. The third and fourth gills were removed and fixed in 2.5% glutaraldehyde, buffered in Na-cacodylate-HCl, pH 7.4.

For thin-section electron microscopy, small pieces of gill tissue containing up to 4 filaments were postfixed in 2% OsO_4 in Na-cacodylate-HCl buffer or in OsO_4-ferrocyanide solution (Karnovsky 1971), dehydrated in a graded series of ethanols and embedded in Agar-Araldite CY 212. Thin sections were cut on an LKB 4800-III Ultrotome and stained with uranyl acetate and lead citrate.

For freeze-fracture electron microscopy, tissue samples were cryoprotected in 30% glycerol in Ringer's solution for 1–2 h at room temperature, mounted on specimen holders, rapidly immersed in the liquid supernatant of melting Freon 22, and stored in liquid N_2. Replicas were produced in a BA 360 M Balzers freeze-fracture apparatus, equipped with a QSG 201 quartz crystal thin-film monitor and an EVM 052 electron beam gun (all Balzers, Liechtenstein) at $-100°$ C and 2×10^{-6} Torr. The replicas were cleaned in commercial bleach, chromic acid and distilled water and mounted on Formvar-coated 400-mesh copper grids.

Thin sections and freeze-fracture replicas were examined in a Siemens Elmiskop IA electron microscope, operated at 80 kV.

The number of strands forming the zonulae occludentes was counted on micrographs printed at a final magnification of 40000:1. Measurements were taken at distances of 20 mm (= 0.5 µm). Extensions of zonulae occludentes at regions where three cells meet were excluded from the measurements (Claude and Goodenough 1973).

Results

In the gills of young adult *Geotria australis*, the chloride cells form extensive multicellular complexes in the interlamellar region of the filament and on the lower part of the filament where lamellae are absent (Bartels et al. 1990). These chloride cells possess (1) membranous tubules representing an extensive intracellular amplification of the basolateral plasma membrane (tubular system), (2) numerous mitochondria, and (3) small vesicles in the apical region of the cytoplasm (Figs. 1, 4). The location and ultrastructural characteristics outlined above are essentially the same as those described for adults of *Lampetra fluviatilis*, *Lampetra japonica* and *Petromyzon marinus* (Nakao 1974, 1977; Youson and Freeman 1976; Peek and Youson 1979a; Bartels et al. 1990). In contrast to the chloride cells of teleosts, those of lampreys do not possess an apical crypt (Copeland 1948; Philpott and Copeland 1963; Hossler et al. 1985) and are not associated with so-called accessory cells (Hootman and Philpott 1980).

Freshwater animals

Thin sections showed that most of the chloride cells (64%) in the gills of those young adult *Geotria australis* that had been retained in freshwater were covered by flange-like extensions of nearby pavement cells (Fig. 1a). However, when adjacent chloride cells were exposed to the external environment, they were frequently separated by a thin process of a pavement cell in the region of the zonula occludens (Fig. 1a, b). The junctions between chloride cells and pavement cells and between neighbouring chloride cells were always extensive (200–250 nm) (Figs. 2a, 3a).

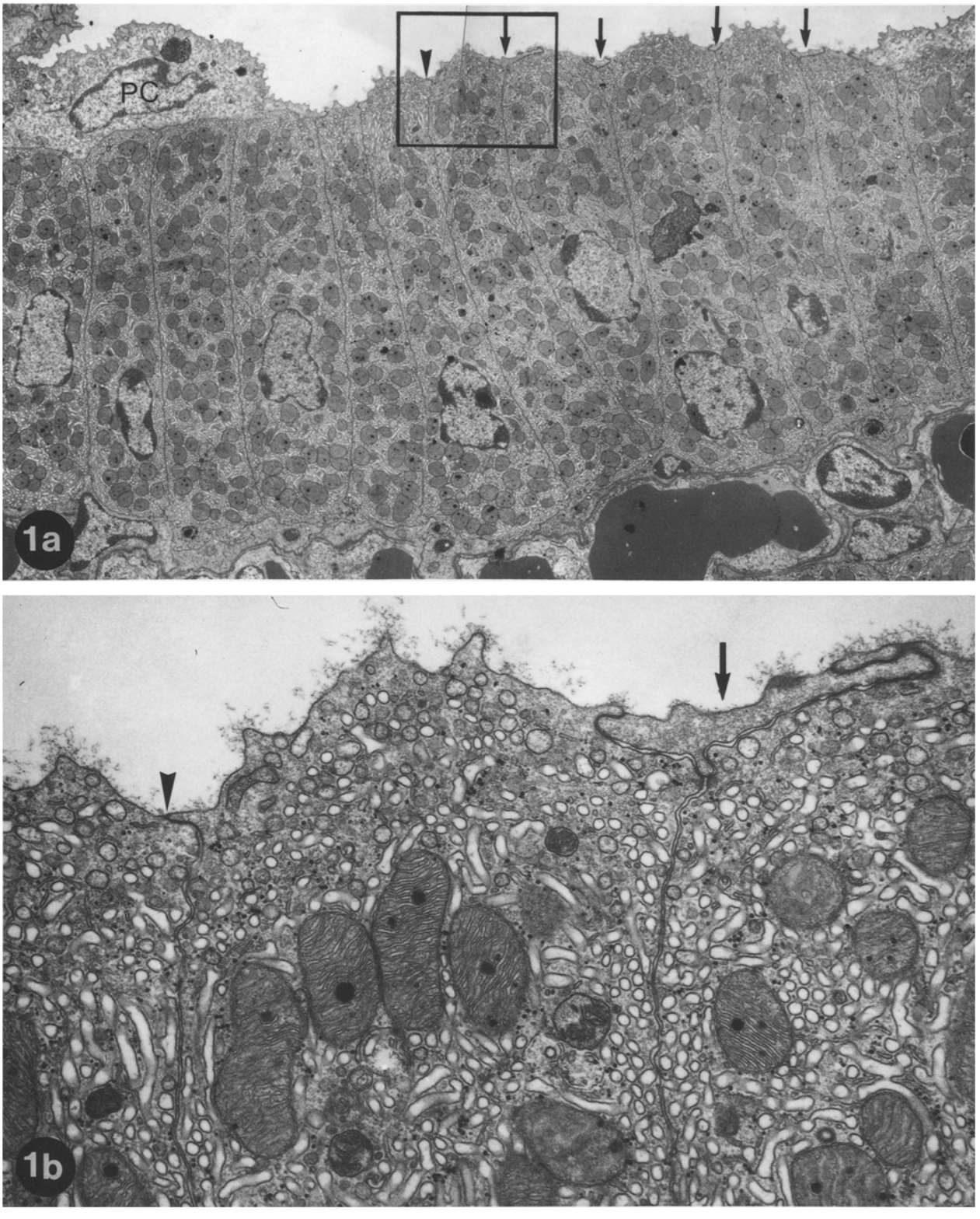
In freeze-fracture replicas, the zonulae occludentes between the chloride and pavement cells and between adjacent chloride cells, were formed by a network of 4 superimposed strands (range 3–7) (Table 1; Figs. 2b, 3b, c).

Seawater animals

The relationship between chloride cells and pavement cells in young adult *Geotria australis* that had been acclimated to full-strength seawater differed conspicuously from those described for animals held in freshwater. For

Table 1. Number of junctional strands of chloride cell junctions in the gill epithelium of young adult *Geotria australis*. Data are expressed as median and ranges (in brackets); *n*, number of measurements

	Occluding junctions	
	Between chloride cells	Between chloride cells and pavement cells
Freshwater	4 (3–6) ($n=9$)	4 (3–7) ($n=80$)
Seawater	1 (1–2) ($n=93$)	3 (2–6) ($n=96$)



Figs. 1–3. Chloride cells in the gills of young adult *Geotria australis* held in freshwater

Fig. 1. a Cross section of a gill filament. Chloride cells are covered by pavement cells (*PC*) at extreme *right* and *left*. The intervening chloride cells are exposed to the environment and, in most cases, the neighbouring chloride cells are separated by pavement cell processes (*arrows*) at the level of the zonulae occludentes. The *arrowheads* point to zonulae occludentes between chloride cells. **b** Apical regions of three chloride cells. The central chloride cell is separated from the one on the right by a pavement cell process (*arrow*), whereas it shows a zonula occludens (*arrowhead*) with the cell on the left. **a** ×4000; **b** ×24000

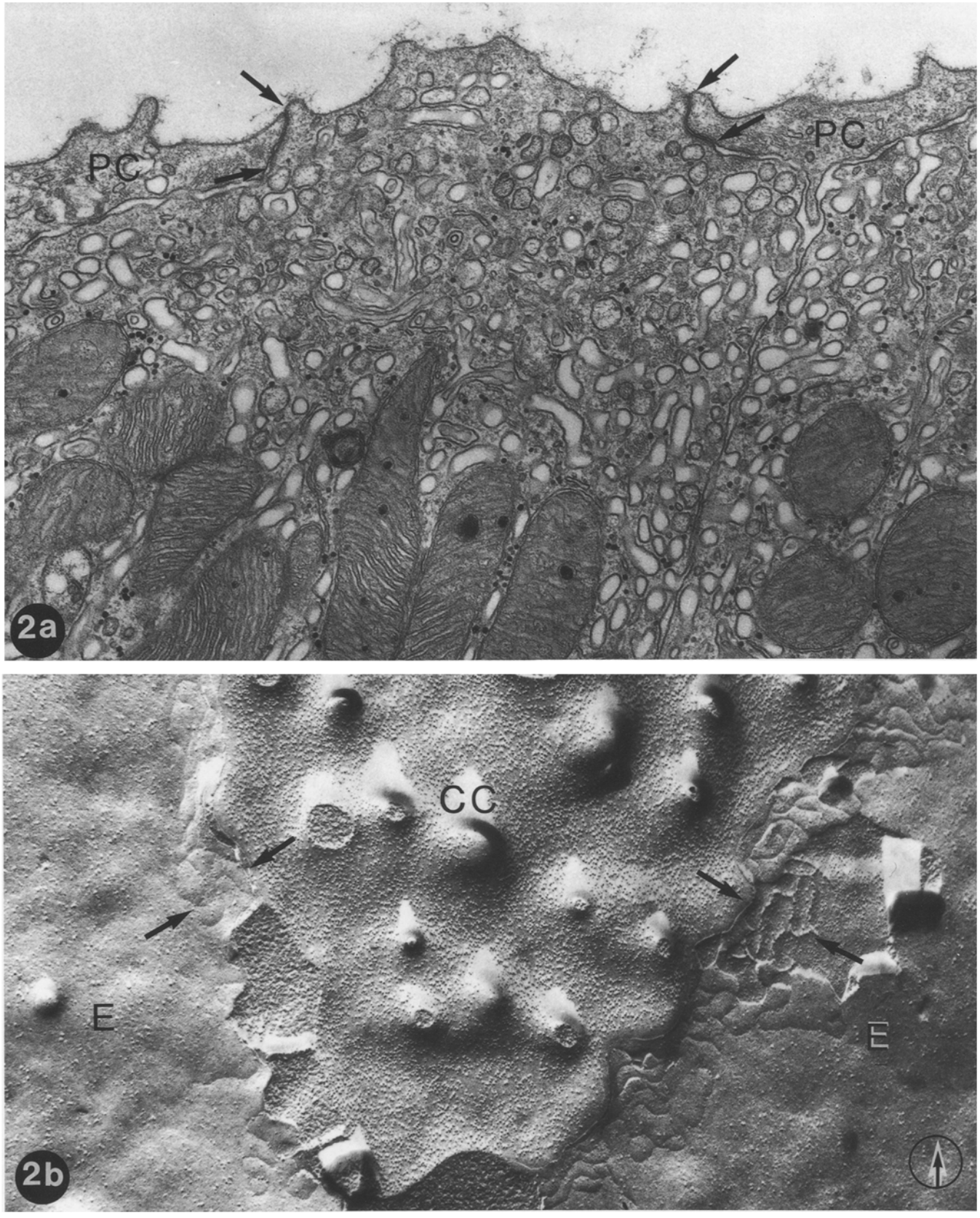


Fig. 2. a Thin section and **b** freeze-fracture replica showing a chloride cell in the centre, linked on either side to pavement cells (*PC*) by extensive zonulae occludentes (between *arrows*), which are formed by a network of strands (**b**). *CC* P-face of the apical membrane of the chloride cell; *E* E-face of the lateral membrane of the pavement cells. In this and the following freeze-fracture electron micrographs the direction of shadowing is indicated by an *encircled arrow* in the right lower corner. **a** ×24000; **b** ×48000

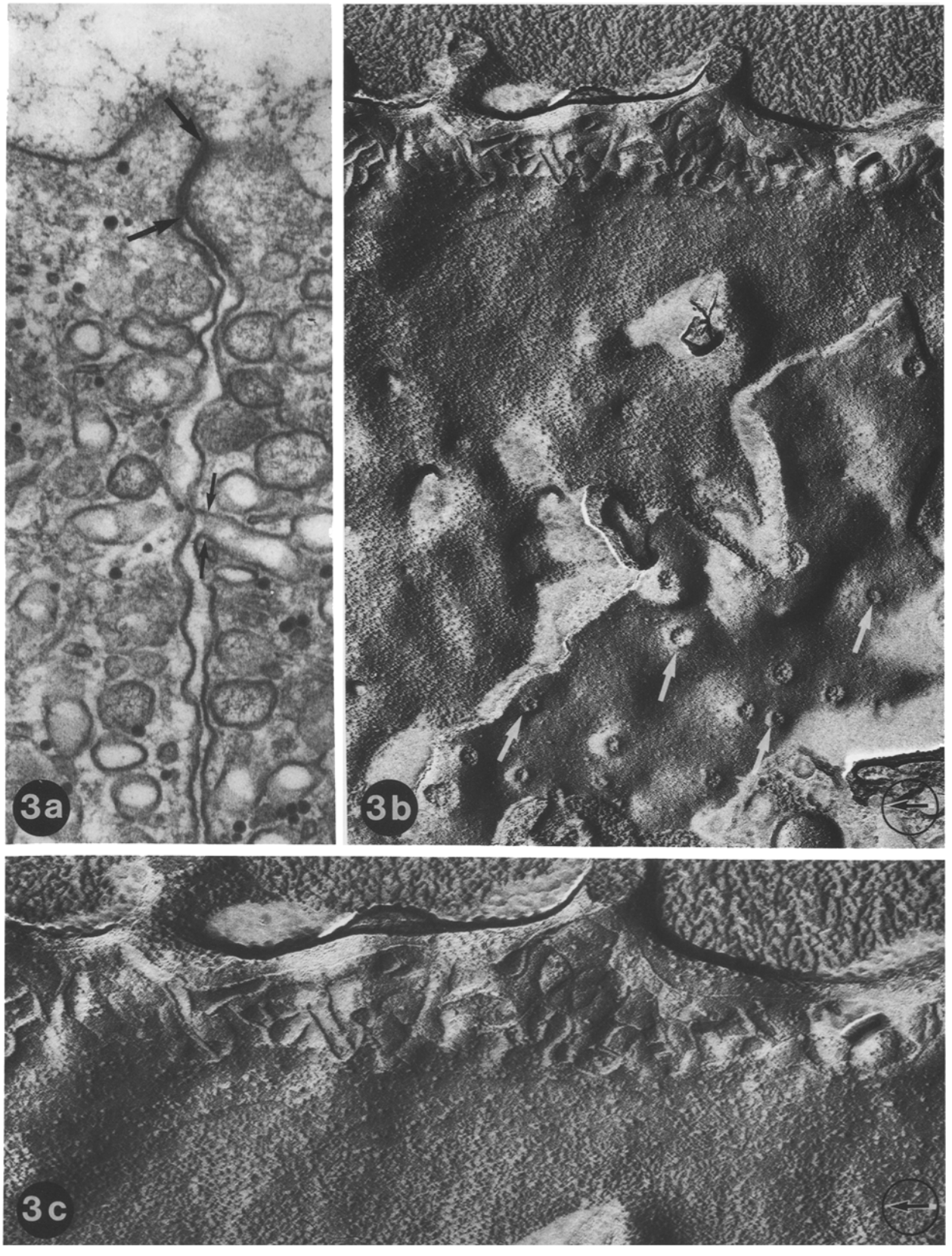


Fig. 3a–c. Zonulae occludentes between adjacent chloride cells. **a** Thin section showing the extent of the junctions between *large arrows*. *Small arrows* denote an opening of the tubular system. **b** Freeze-fracture replica showing the network of strands that comprises the zonula occludens. *Arrows* point to openings of the tubular system. **c** Higher magnification of **b**. **a** ×80000; **b** ×48000; **c** ×88000

example, the proportion of chloride cells not covered by pavement cells had risen from 36% to 71% (compare Figs. 1a and 4a). Furthermore, those chloride cells that were exposed to the environment were now no longer separated by finger-like processes of pavement cells (Fig. 4). This resulted in a marked increase in the number of chloride cells that were linked directly by zonulae occludentes. Thin sections demonstrated that these junctions were shallow (25–40 nm) (Figs. 4b, 5a), whereas those between chloride and pavement cells remained extensive (~250 nm) (Fig. 5b).

In freeze-fracture replicas, the zonulae occludentes between chloride cells comprised only 1 strand or 2 parallel strands (Fig. 5c). The zonulae occludentes between chloride and pavement cells were on average formed by a network of approximately 3 superimposed strands (range 2–6) (Table 1; Fig. 5d).

Discussion

Chloride cells do not develop in the gills of anadromous lampreys until the ammocoete metamorphoses into the adult and they disappear after the animal re-enters freshwater on its spawning migration, following the completion of the marine trophic phase (Morris 1957; Morris and Pickering 1975, 1976; Youson and Freeman 1976; Peek and Youson 1979b). The current investigation has shown that approximately two thirds of the chloride cells of downstream-migrating *Geotria australis* are covered by pavement cells. Furthermore, many of these cells subsequently become exposed to the environment when the animal enters seawater. It is thus concluded that, in lampreys, the chloride cells only play a crucial role in osmoregulation during the marine phase of the life cycle.

Chloride cells in seawater

The present study has shown that the zonulae occludentes between adjacent chloride cells in seawater-acclimated lampreys are shallow and consist of only 1 or 2 strands. This situation, which is characteristic of leaky epithelia (Claude and Goodenough 1973; Claude 1978; Ernst et al. 1981), parallels that described for the chloride cells of teleosts in marine environments (Sardet et al. 1979; Ernst et al. 1980; Hwang and Hirano 1985; Hwang 1987; Pisam et al. 1988). From a combination of ultrastructural and electrophysiological studies on the chloride cells in the flat opercular epithelium of the seawater-acclimated killifish, *Fundulus heteroclitus*, Ernst et al. (1980) concluded that these junctions provide the low-resistance pathways by which Na^+ passively enters the external environment. The absence in lampreys of a flat epithelium that is rich in chloride cells has prevented similar ion flux studies being carried out with this group. However, the basic similarity between the fine structure, multicellular arrangement and junctional organisation of their chloride cells and those of teleosts in marine environments implies that a similar mechanism for the hypertonic secretion of excess NaCl is present in the gills of lampreys. This mechanism is thus likewise envisaged as involving a transcellular transport of Cl^-, coupled to the activity of the Na^+/K^+-ATPase in the tubular system, and a paracellular and passive transport of Na^+. Since adult lampreys, like teleosts, overcome osmotic losses of water in marine environments by swallowing saltwater that is markedly hypertonic to their body fluids (Morris 1958; Pickering and Morris 1970), they also require such a mechanism for removing the resultant excess of NaCl that enters the body.

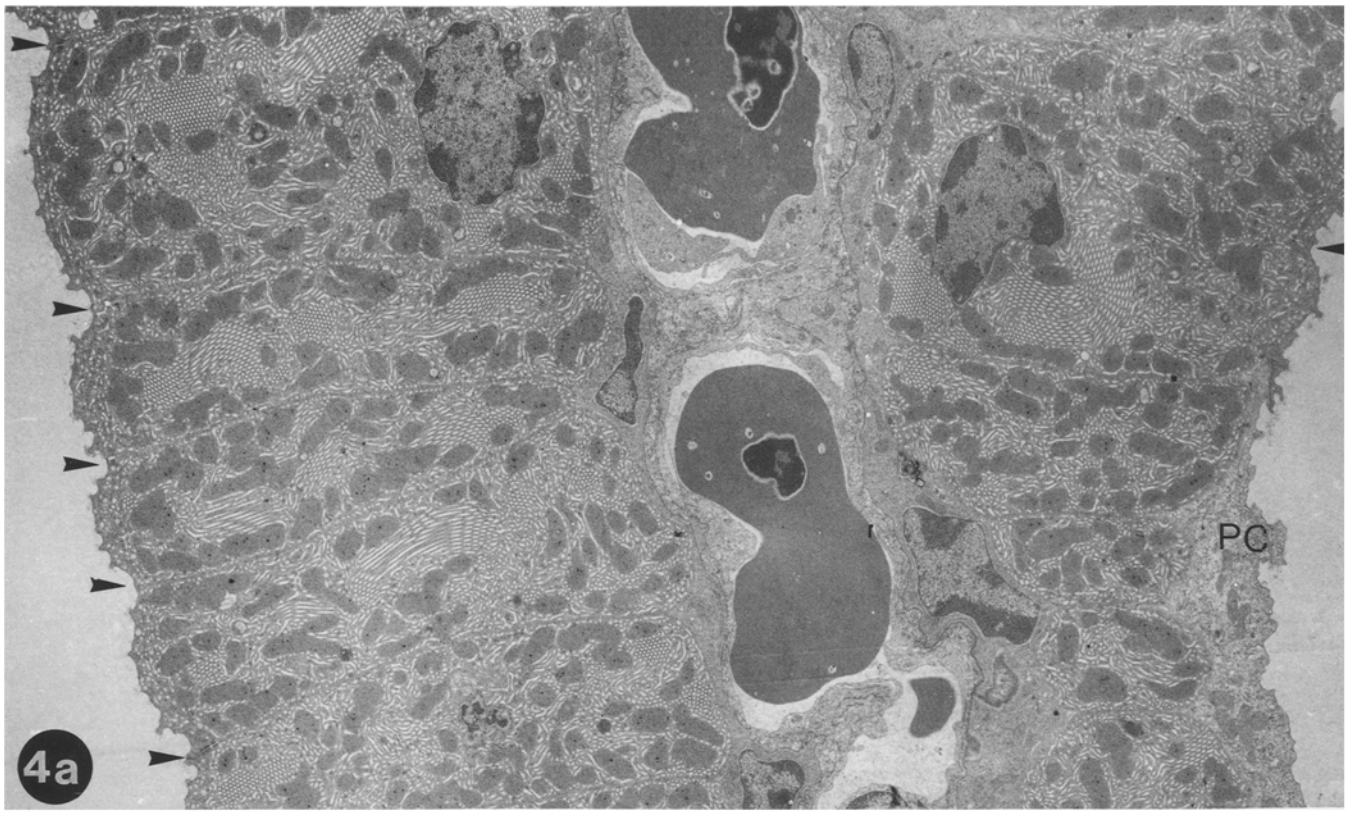
Chloride cells in fresh water

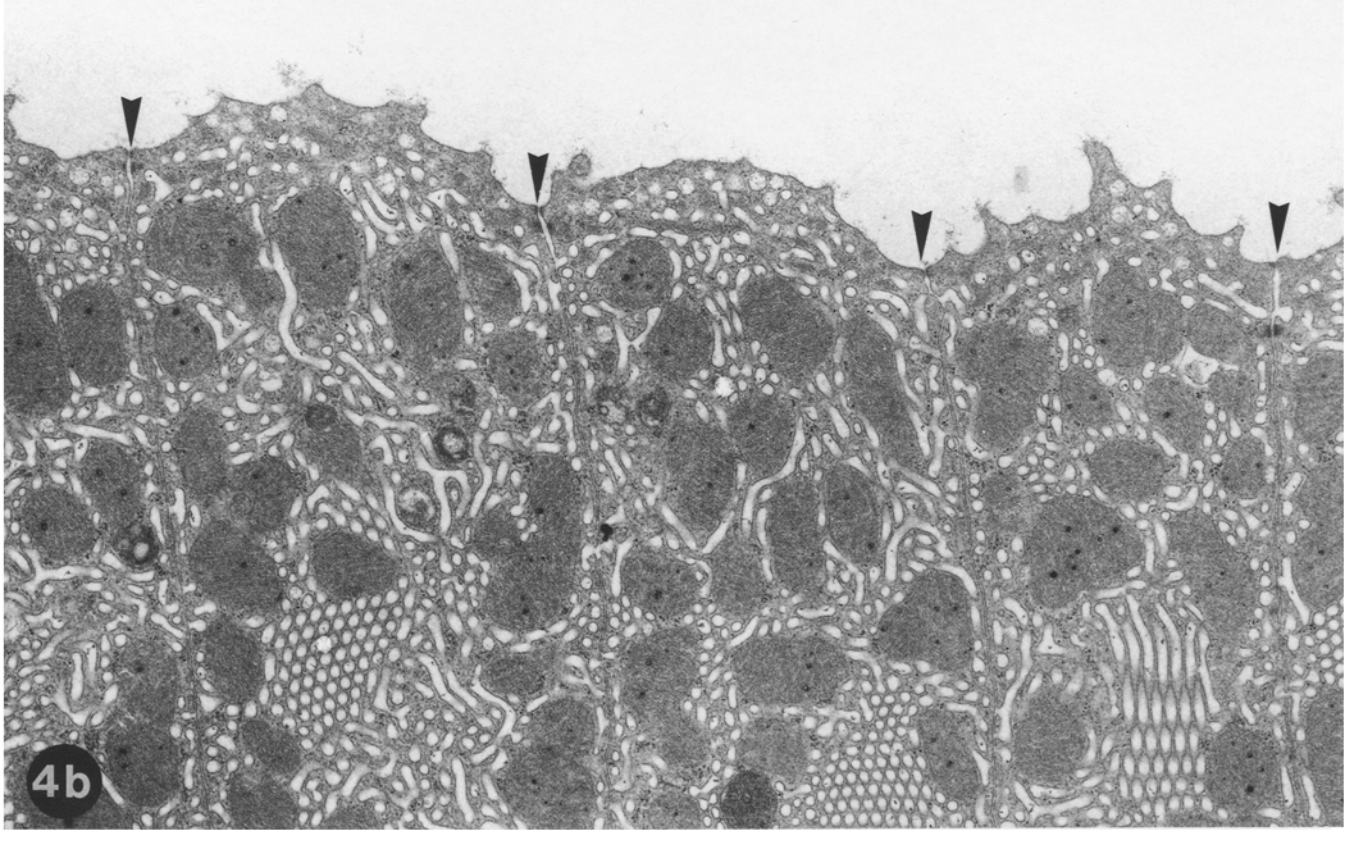
Although the extensive arrangements of leaky junctions in the gill epithelium of adult lampreys are considered to play a crucial role in ion transport in seawater, such structures would be detrimental to the same animals prior to the time that they enter the sea. This conclusion is based on the fact that a leaky paracellular pathway would reduce the ability of the animal to conserve ions at a time when it is living in a markedly hypotonic environment. The present study has shown that, at the level of the zonulae occludentes, pavement cell processes frequently separate those chloride cells in which the apical surfaces are exposed to the environment. The junctions between chloride and pavement cells are extensive and complex, as are those junctions between chloride cells that are not separated by pavement cells. Such junctions are assumed to impede the passive movement of ions to a greater extent than those that characterise the paracellular pathway between chloride cells in seawater-acclimated lampreys.

It is also relevant that most of the chloride cells of young adult *Geotria australis* held in freshwater are covered by pavement cells. This covering, which parallels that in comparable stages of *Petromyzon marinus* and *Lampetra fluviatilis* (Peek and Youson 1979a; own unpublished results), prevents chloride cells from transporting Cl^- and Na^+ across the gill epithelium. Since the apical membrane of the pavement cells of lamprey gills is assumed to have a low permeability to water (Bentley 1962; Bartels 1989), the presence of pavement cells over much of the gill surface would also help to reduce the osmotic influx of water.

Transition from freshwater to seawater

From the above account and discussion, it is evident that the transition of young adult lampreys from freshwater to saltwater is accompanied by the retraction of the flanges and processes of the pavement cells. The area of the apical membrane of the chloride cell and the number and length of the zonulae occludentes between the chloride cells thus become greatly increased. Concomitantly, all pre-existing and new zonulae occludentes between chloride cells become characterised by





Figs. 4, 5. Chloride cells in the gills of *Geotria australis* acclimated to full-strength seawater

Fig. 4. a Lower-power and **b** high-power micrograph of a cross-sectioned gill filament. These chloride cells are not separated by pavement cell processes at the level of the zonula occludens (*arrowheads*). Note that the cytoplasmic tubules form bundles and are more regularly arranged than in freshwater lampreys. *PC* Pavement cell. **a** ×5500; **b** ×18000

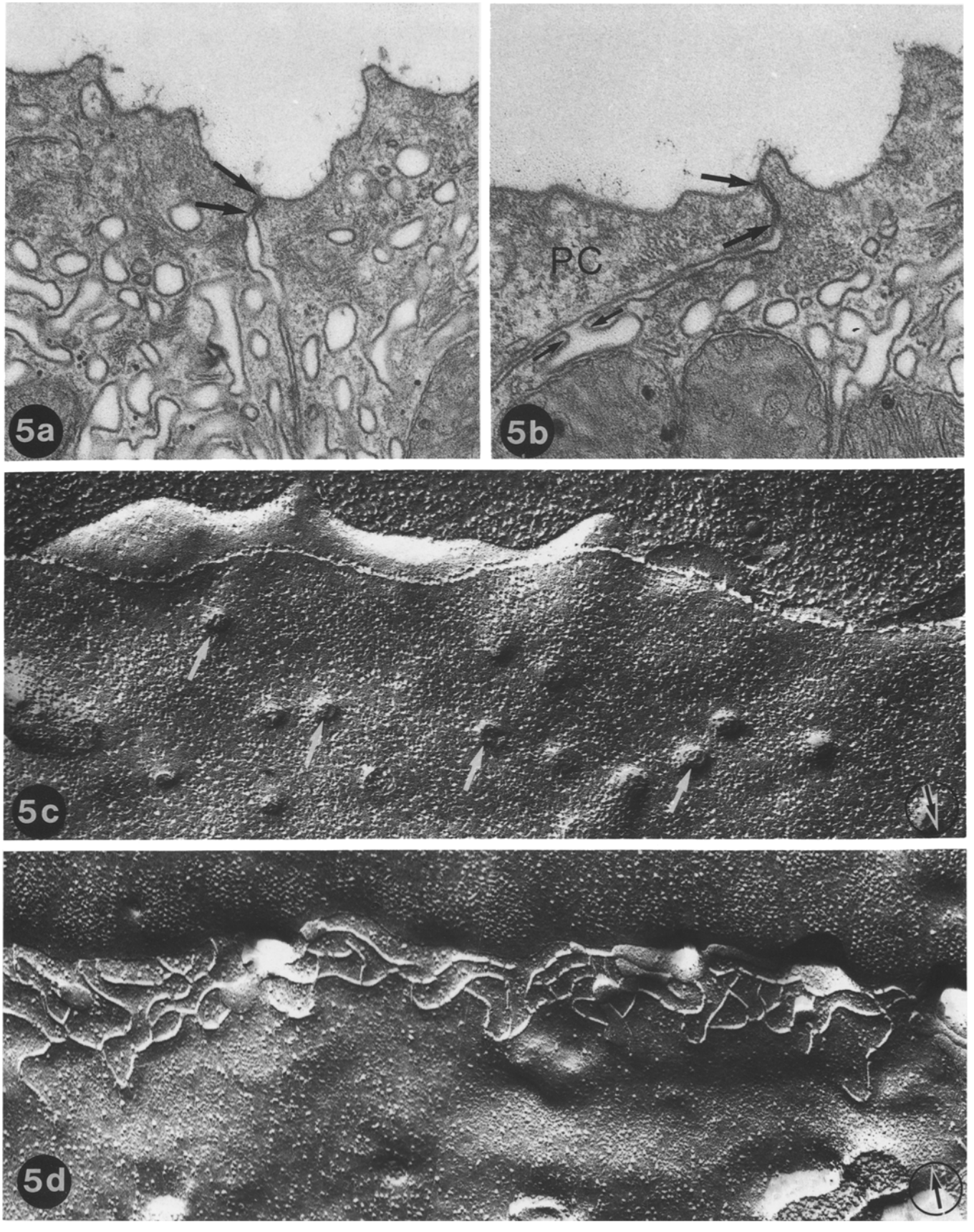


Fig. 5a–d. Thin sections and freeze-fracture replicas of shallow zonulae occludentes between adjacent chloride cells (**a, c**) and of extensive zonulae occludentes between a chloride cell and a pavement cell (**b, d**). *Large arrows* in **a** and **b** denote the extent of the zonulae occludentes, *small arrows* in **b** and *white arrows* in **c** the openings of the tubular system. **a** ×70000; **b, c** ×80000; **d** ×60000

the presence of only 1 or 2 strands. These changes produce a more leaky paracellular pathway.

The basic structure of the chloride cell has developed by the end of metamorphosis. Furthermore, both the retraction of the pavement cells and their processes and the production of shallow occluding junctions can occur rapidly. The gill of young adult lampreys is thus equipped to cope with the osmotic problems posed by a hypertonic environment at the time the marine phase is initiated.

Such a view is consistent with the fact that young adults of anadromous lampreys can frequently be transferred directly from freshwater to full-strength seawater (Potter and Huggins 1973; Potter and Beamish 1977; Potter et al. 1980). The ability of these lampreys to acclimate rapidly to hypertonic environments is almost certainly related in part to the functioning of (1) a subepithelial nerve plexus which is in close spatial relationship with the chloride cells at the base of the filament (Bartels et al. 1990), and (2) the communicating (gap) junctions that develop between the chloride cells while the lamprey is still in freshwater (Bartels and Potter 1990). Since these junctions allow the bidirectional exchange of ions and small metabolites between the coupled cells (Peracchia 1980; Hooper and Subak-Sharpe 1981), they can efficiently support the onset of chloride cell function in a hypertonic environment by synchronising their secretory activity.

Comparisons between chloride cells of lampreys and teleosts

The fact that in full-strength seawater the osmotic pressure of the blood of both teleosts and lampreys is far lower than that of the environment has been taken as reflecting a long prior history of these groups or their ancestors in freshwater (Pickering and Morris 1970; Lutz 1975; Beamish et al. 1978). Pickering and Morris (1970) have pointed out that the lineages leading to contemporary teleosts and lampreys have been separated for at least 500 million years, and have concluded that their "almost identical" marine osmoregulatory mechanisms have evolved independently. Such an independent evolution would be consistent with the fact that, although the chloride cells of both groups possess certain basic common characteristics, their pattern of development, arrangement and morphology differ in the respects listed below.

The changes that occur during lamprey metamorphosis as a preparation for life at sea can be compared with those that take place during the parr-smolt transformation (smoltification) of anadromous salmonids (Folmar and Dickhoff 1980). However, whereas the gills of the initial freshwater stage in the life cycle of lampreys (ammocoetes) do not possess chloride cells (Morris and Pickering 1975; Youson and Freeman 1976), those of the corresponding stage in salmonids (parr) contain two sub-types of such cells (Pisam et al. 1988). The predominant type of these chloride cells increases in size and complexity during smoltification into a cell that corresponds to the chloride cell of euryhaline teleosts in seawater.

In seawater, the chloride cells of both lampreys and teleosts are arranged in multicellular complexes. However, in lampreys, each of these complexes typically comprises a large number of cells that form a continuum extending from the base of the filament into the interlamellar regions, whereas in teleosts the complexes are far more numerous but consist of only 2–4 cells (Philpott and Copeland 1963; Sardet et al. 1979; Karnaky 1986; King et al. 1989). The chloride cells in teleosts are often associated with smaller accessory cells within the multicellular complexes (Hootman and Philpott 1980; Chretien and Pisam 1986; Hwang 1987; Pisam et al. 1989, 1990). The accessory cells do not have the characteristics of mature chloride cells, such as an extensive tubular system and associated Na^+/K^+-ATPase activity (Hootman and Philpott 1980), and they contain less cytochemically demonstrable carbonic anhydrase (Lacy 1983). Such cells have never been observed in the gills of lampreys. Furthermore, adjacent chloride cells (and also chloride and accessory cells) interdigitate and share an apical crypt in teleosts, but not in lampreys. Finally, freeze-fracture studies have revealed that, in contrast to the situation in teleosts in seawater, the apical membrane of the chloride cells of seawater-acclimated lampreys contains prominent clusters of particles (cf. Sardet et al. 1979; Ernst et al. 1980; Bartels et al. 1987).

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