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Electron microscopical and histochemical evidence of chloride cells in tracheal gills of mayfly larvae

Elektronenmikroskopischer und histochemischer Nachweis von Chloridzellen in der Tracheenkiemen von Eintagsfliegenlarven

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Abstract

In the tracheal gill epithelium of mayfly larvae a special cell type has been observed that resembles the chloride cells of teleost gills and has, therefore, been termed ephemerid chloride cell.

In *Rhithrogena semicolorata* the chloride cells are single cells, which are characterized by numerous mitochondria, an apical labyrinth of tubular invaginations of the plasma membrane, and an apical cavity with microvilli. There is also a porous plate above the apical cavity that is a local differentiation of the cuticle.

In *Cloeon dipterum* the chloride cells are organized into complexes. The central cell of each chloride cell complex possesses a cone-shaped apex which is extended into a funnel-shaped recess of the cuticle in direct apposition to the porous plate. The surrounding cells are extensively interdigitated, thus forming a basal labyrinth.

Histochemical tests for sodium and chloride indicate that a high concentration of these ions is localized within the porous plate and apical cavity in *Rhithrogena,* and within the porous plate and central cell apex in *Cloeon.*

The results suggest that the ephemerid chloride cells are involved in the osmoregulation of these animals and that their main function is probably the absorption of electrolytes.

Introduction

In continuation of our studies on the fine structure of the tracheal gill epithelium of fresh water insect larvae [34], we have observed a special type of cells in mayfly larvae which are distinctly different in fine structure from the other cells constituting the respiratory epithelium. The ultrastructural features of these cells are very similar to

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other cells which are known to be involved in ion transport and osmoregulation. Since a review of the literature revealed no references describing osmoregulation in the tracheal gills of mayfly larvae [2, 16, 23, 24, 29, 31, 36, 37], these cells were studied in two ephemerid species in order to get further indications to their possible function.

Materials and methods

The nymphs of two different European ephemerid species were used: *Cloeon dipterum* L. *(Baetidae)* living in stagnant water, and *Rhithrogena semicolorata* CURT. *(Ecdyonuridae)* living in running water. Their tracheal gills were fixed for 2 hours in 2 % OsO_4 and 1 % $K_2Cr_2O_7$, *p*H 7.2 [38], subsequently dehydrated in graded alcohols, stained wih uranyl acetate and phosphotungstic acid during dehydration [38], and embedded in styrene methacrylate [17]. Ultrathin sections were cut with a LKB ultramicrotome and studied with a Philips electron microscope EM 200. One micron thick sections from the same materials were used for light microscopy.

To evaluate the suggested function of the cells in question, the tracheal gills of both species were fixed with 1 % OsO_4 and 2 % K $[Sb(OH)_6]$, *p*H 8.5, for histochemical precipitation of sodium]11[, or with 1.5 % osmium tetroxide and 1 % silver lactate in 0.1 M cacodylate-acetic buffer, *p*H 6.4, for histochemical demonstration of chloride [11, 13]. In the latter case fixation was performed under red safe-light, and the fixed gills were rinsed in a nitric acid bath during dehydration [12]. The precipitate resulting from fixation with the osmium/silver solution was identified by selected area electron diffraction.

Results

For simplicity, the cells studied herein shall be termed chloride cells because they are closely analogous to the chloride cells in the gills of fish.

Distribution of the ephemerid chloride cells. The ephemerid chloride cells are irregularly distributed within the epithelium of the tracheal gills (fig. 1 a), that adhere to the abdominal segments [9]. They are concentrated predominantly in the basal and middle regions of the gills. In the nymphs of *Cloeon dipterum* there are two pairs of plate-like tracheal gills attached to each of the first seven abdominal segments. The lower gill, the one closest to the body, of each pair is larger and seems to be richer in chloride cells than the upper gill. The nymphs of *Rhithrogena semicolorata* have the same number and distribution pattern of tracheal gills as *Cloeon*. In contrast to *Cloeon* however, only the lower gill of each pair is lamellate, whereas the upper one is filamentous and appears as a tuft on the side of each segment. In the plate-like tracheal gill of this species the chloride cells are distributed in essentially the same pattern as they are in the lower plate-like gills of *Cloeon*. The distribution and number of chloride cells in the filiform gills have not been well established, because only a few sections of these gills were studied.

Fine structure of the chloride cells in Rhithrogena. The epithelium of tracheal gills is interspersed with tracheols just below the cuticle and, therefore, seems to be mainly respiratory in function [37]. The chloride cells of this species (fig. 1 b) are observed as single cells irregularly distributed within the epithelium. Their fine structure differs markedly from the other epithelial cells. They are roughly spherical to pyramidal in shape and the nucleus is usually located in the basal part of the cell. The cells rest on a basal lamina bordering the central hemolymph space of the tracheal gill.

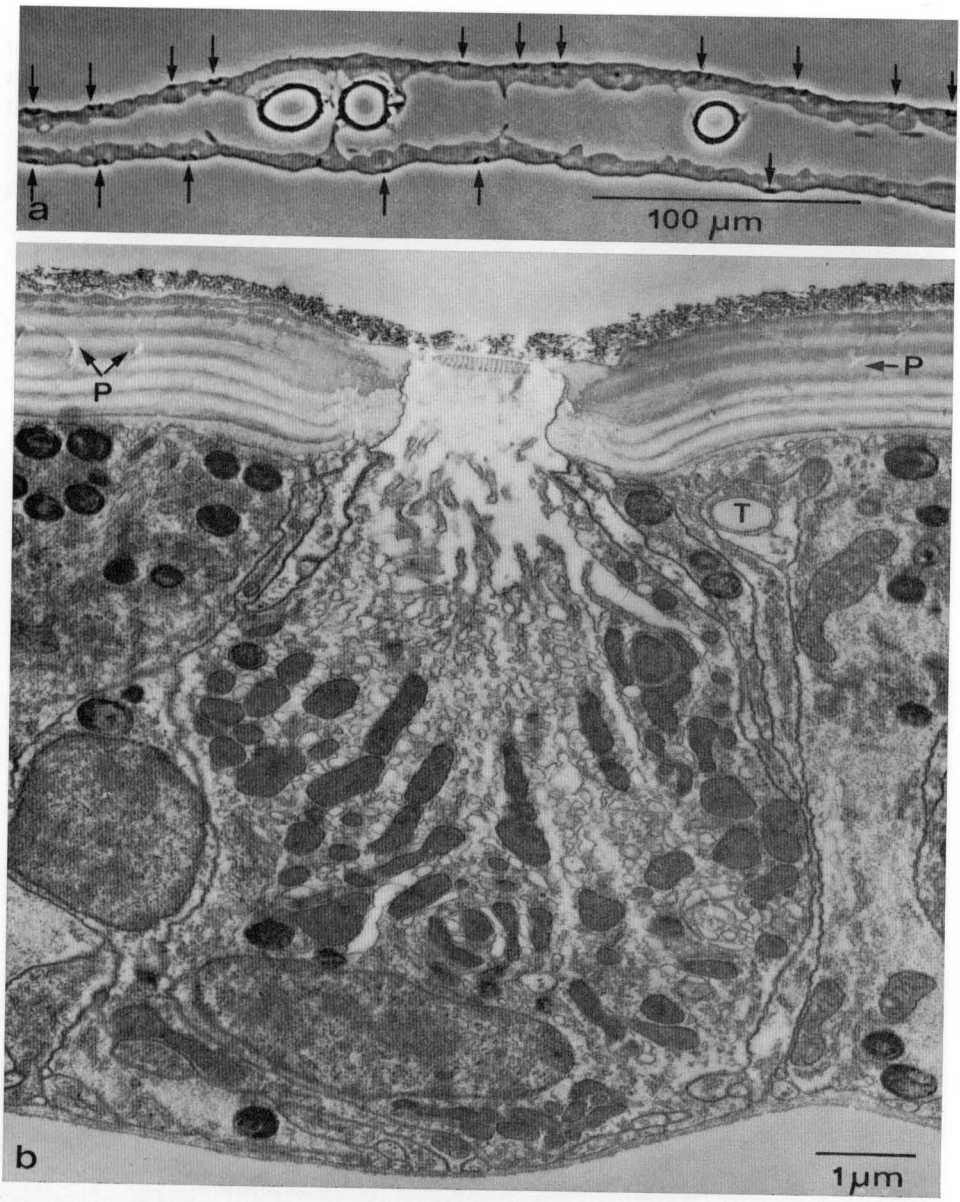
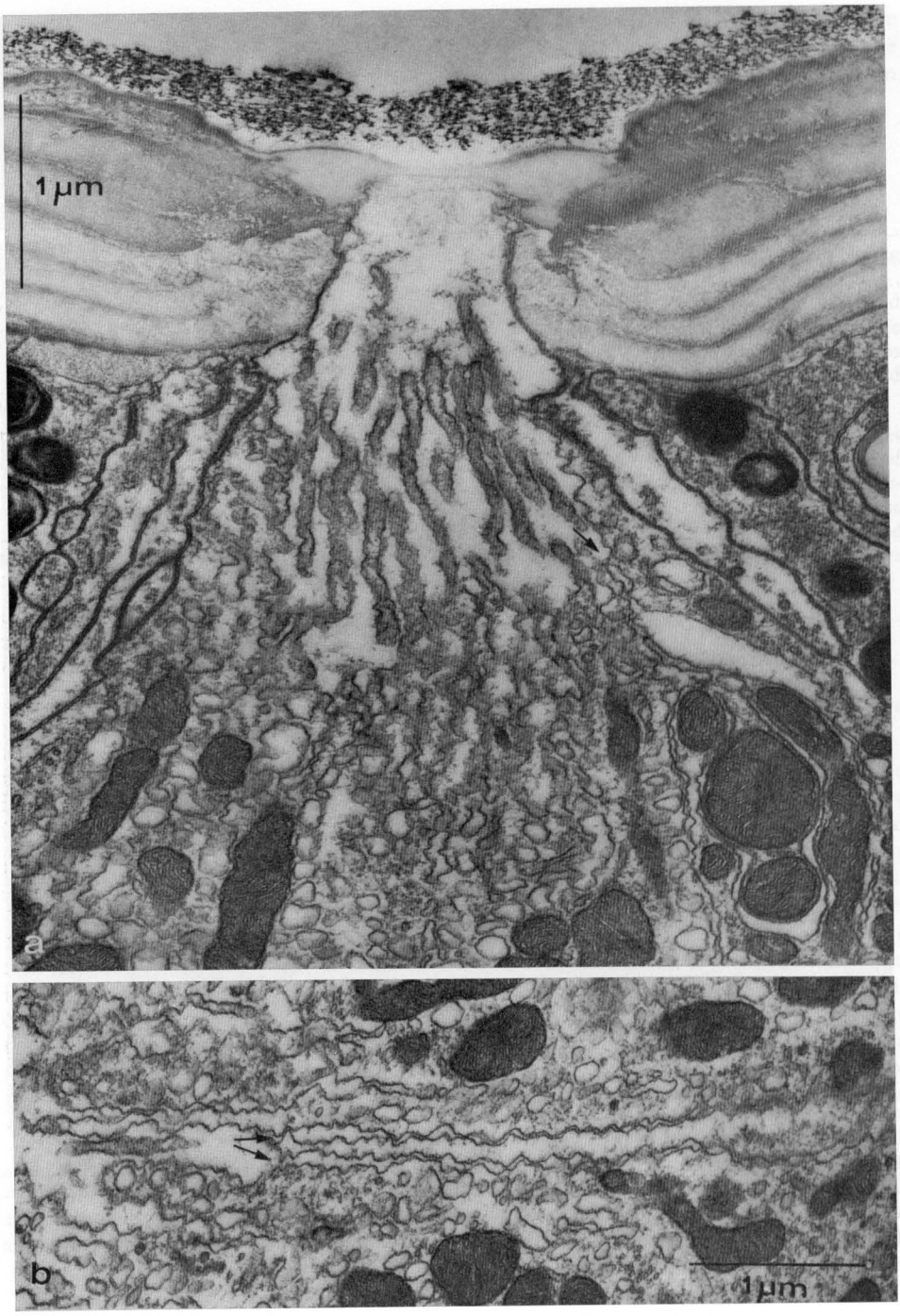


Fig. 1a. One micron thick cross section through the middle region of a tracheal gill of *Cloeon dipterum*, showing distribution of chloride cell complexes (*arrows*). Phase contrast. 360 ×. – **b.** Chloride cell within the epithelium of a lamellate tracheal gill of *Rhithrogena semicolorata*. The connection of the porous plate with the normal cuticle has been broken during ultrathin sectioning. T Tracheol. P normal pore channels within the cuticle. 13,200 ×.


1 µm
a
b
1 µm

The basal part of the chloride cells shows a poorly developed basal labyrinth [22] that appears to be created by interdigitating processes from adjacent cells of the respiratory epithelium as indicated by the fine structure of the mitochondria. The mitochondria within these cell processes correspond to the mitochondria of the respiratory epithelial cells and differ from the mitochondria of the chloride cells (fig. 1 b, 3). The lateral cell boundaries are fairly smooth and generally flanked by flattened cellular sheets, which also belong to cells of the respiratory epithelium.

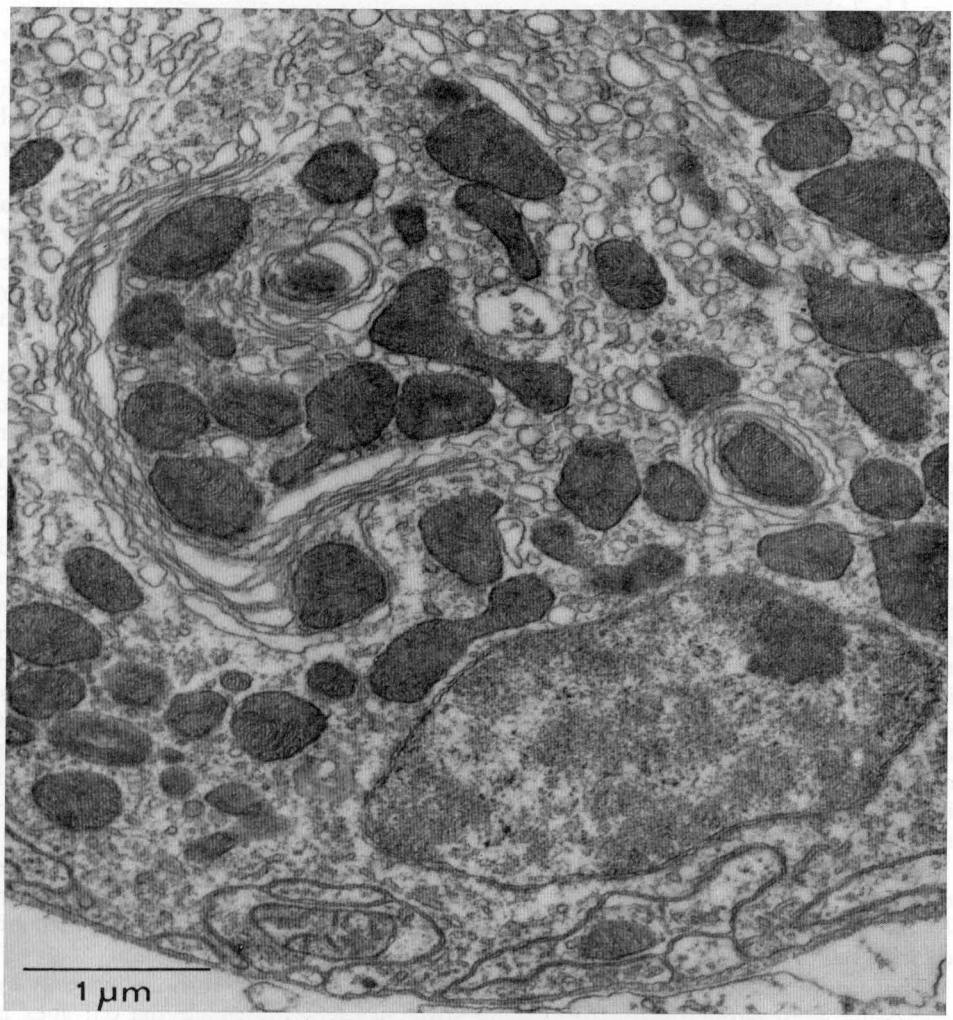


Fig. 3. Intermediate and basal part of a chloride cell of *Rhithrogena,* showing Golgi complex and poorly developed basal interdigitation. 25,200 ×.

Fig. 2 a. Apical cytoplasm of a chloride cell of *Rhithrogena.* The section runs just besides the porous plate. Mitochondrial pump situation in the lower right edge. 27,300 ×. – **b.** Apical cytoplasm with part of the apical cavity (left side) of a chloride cell of *Rhithrogena. Arrows* (see also arrow in fig. 2 a) point to deep invatinations of the apical plasma membrane. 25,500 ×.

The apical part of the chloride cells is slightly constricted and invaginates to form a large apical cavity just beneath the cuticle. The bottom and sometimes also the lateral walls of this cavity give rise to fairly long microvilli, which are somewhat irregular in outline (fig. 1 b, 2 a). Another common feature of these cells is that the cuticle, which is 1 µm thick where it covers the respiratory epithelium, becomes thin very abruptly over the apical cavity of the chloride cells. In this way the lumen of the apical cavity actually extends into a region which is normally occupied by the cuticle (fig. 1 b, 2 a). In other words, there is a large hole in the cuticle above each of the chloride cells. This hole, however, is closed to the outside by a tiny cuticular plate, which is continuous with the thicker part of the cuticle and which exhibits a peculiar cross-striated pattern. This striated plate gives the impression of pores or channels. Both the tiny porous plates and the normal cuticle are covered with a fuzzy coat. A material similar in structure, but much lower in contrast is also found within the apical cavity and associated with the microvilli.

The cytoplasm of the chloride cells is characterized by an abundance of mitochondria and by numerous vesicles and tubules (fig. 1 b, 2 a, b, 3). The mitochondria normally occupy the intermediate and basal parts of the cells. They markedly differ from the mitochondria of the respiratory epithelial cells by their high electron contrast and by their densely packed cristae (fig. 1 b), which presumably is a reflection of their high capacity for oxidative metabolism.

The tubules and vesicles are crowded in the apical part of the chloride cells and extend to the supranuclear region. Numerous vesicles are immediately adjacent to the well-developed Golgi apparatus, which is usually also found in the supranuclear region (fig. 3). As far as the membranous tubules are concerned, most of them seem to originate from invaginations of the plasma membrane between the microvilli of the apical cavity. As clearly shown in fig. 2 a and b *(see arrows)*, the lumina of the tubules are continuous with the extracellular space of the apical cavity. These tubules could often be followed to the supranuclear region. Sometimes a close relationship between tubules and mitochondria could be observed. The organization of these components resembles closely the mitochondrial pump which was described by COPELAND [3] in the epithelium of the mosquito larvae anal papilae. The tortuous tubules follow an irregular course through the cytoplasm and therefore, when cut in cross section, they often appear as isolated vesicles or rows of vesicles. Most of these vesicles and tubules are therefore probably part of a plasma membrane invagination complex constituting an apical labyrinth within the chloride cells. On the basis of the techniques employed it is very difficult to determine, whether this apical ivagination complex is intermingled with elements of the endoplasmic reticulum [compare also 21, 26, 28].

Fine structure of the chloride cell complexes in Cloeon. The chloride cells within the tracheal gill epithelium of *Cloeon* nymphs are structurally different from those in *Rhithrogena* in several respects. The primary difference is that in this species there are chloride cell complexes (fig. 4) rather than single chloride cells.

The central cell of this complex generally contrasts with the surrounding cells by possessing dense ground cytoplasm. At the lower level of the cuticle the cell apex is deeply constricted and extends through a narrow cuticular hole. The apical cytoplasmic extension then expands within the cuticle and forms a cone-shaped plug, which fits exactly into a funnel-shaped recess of the cuticle (fig. 4, 5 a). Therefore no apical cavity exists in the chloride cell complex of this species, because the distal surface of the central cell is actually in contact with the porous plate (fig. 5 a). The distal surface also gives rise to irregularly shaped, tightly packed, short microvillous-like folds.

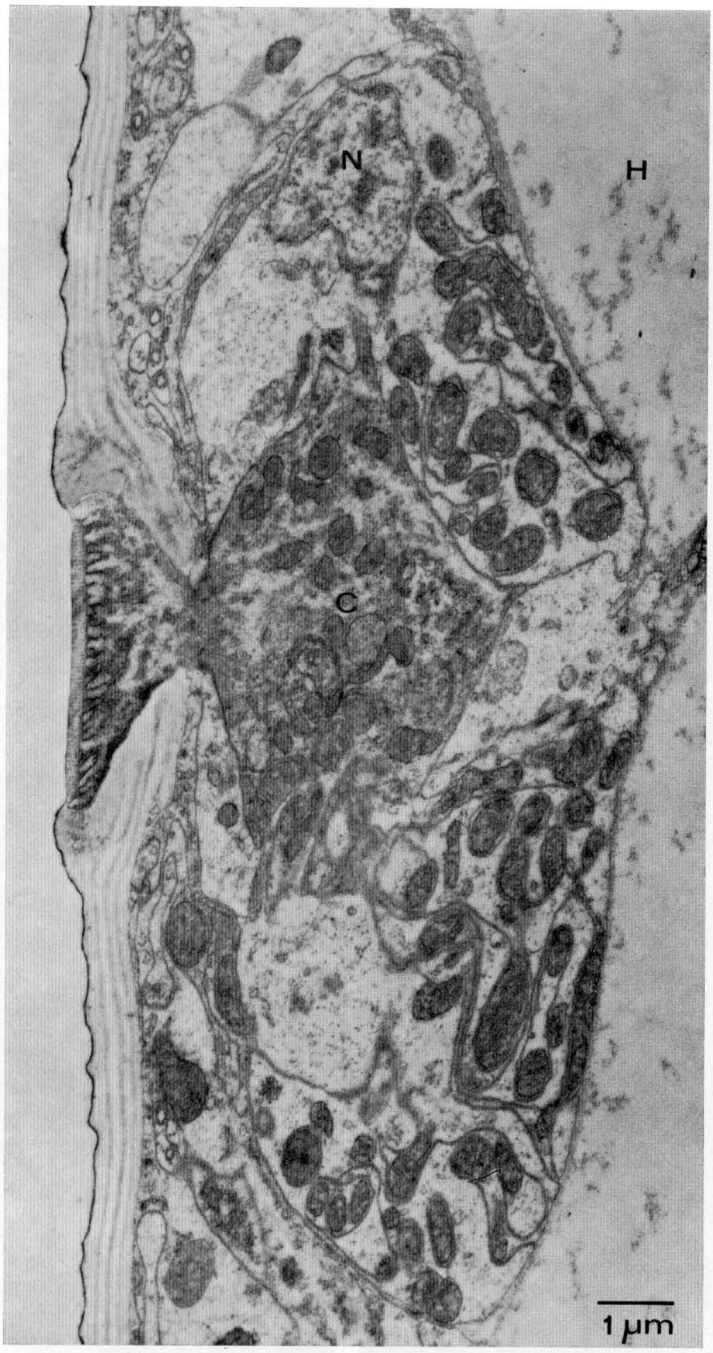
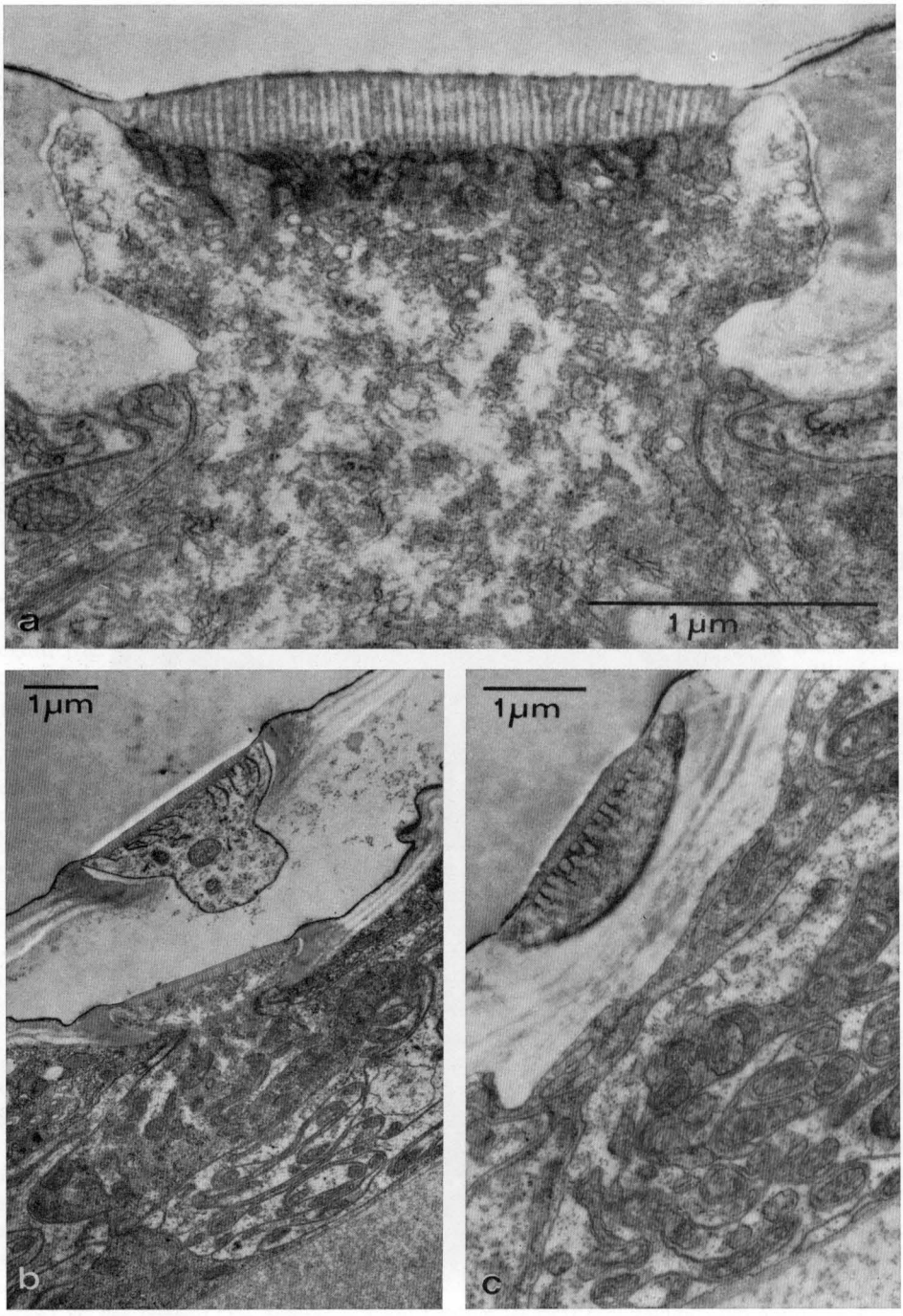


Fig. 4. Chloride cell complex in a tracheal gill of *Cloeon dipterum*. – C Central cell. H Hemolymph space. N nucleus of a surrounding cell. 10,400 ×.


a
1 µm
1µm
b
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c

The porous plate is continuous with the thick part of the normal cuticle in *Cloeon* (fig. 5 a), but it is much larger than in *Rhithrogena*. Therefore, these plates must be regarded as local specializations of the cuticle, which differentiate just over the chloride cells. If the section does not run through the center of the porous plate but through the peripheral part, the cell apex of the central cell erroneously appears to be isolated within the cuticle (fig. 5 c). The adhesion of the cell apex within the funnel-shaped recess seems to be very strong. During a moult, the cell apex does not retract from the hollow, but it is pinched off with the cuticle and appears to be rebuilt (fig. 5 b). The possibility that this is an artifact, which occurs only when the tracheal gills are fixed during the moulting process, can be excluded, because as shown in fig. 5 b, a new apex and a new porous plate have already formed prior to fixation.

The apical cytoplasm of the central cell contains relatively fewer vesicles and tubules than in the chloride cells of *Rhithrogena*. In addition, connections between tubules and the apical plasma membrane were never observed. These structures in *Cloeon* are therefore suggested to be elements of the smooth endoplasmic reticulum.

The central cell is surrounded at its basal and lateral surfaces by additional cells which give the entire chloride cell complex the appearance of being flattened or disc-

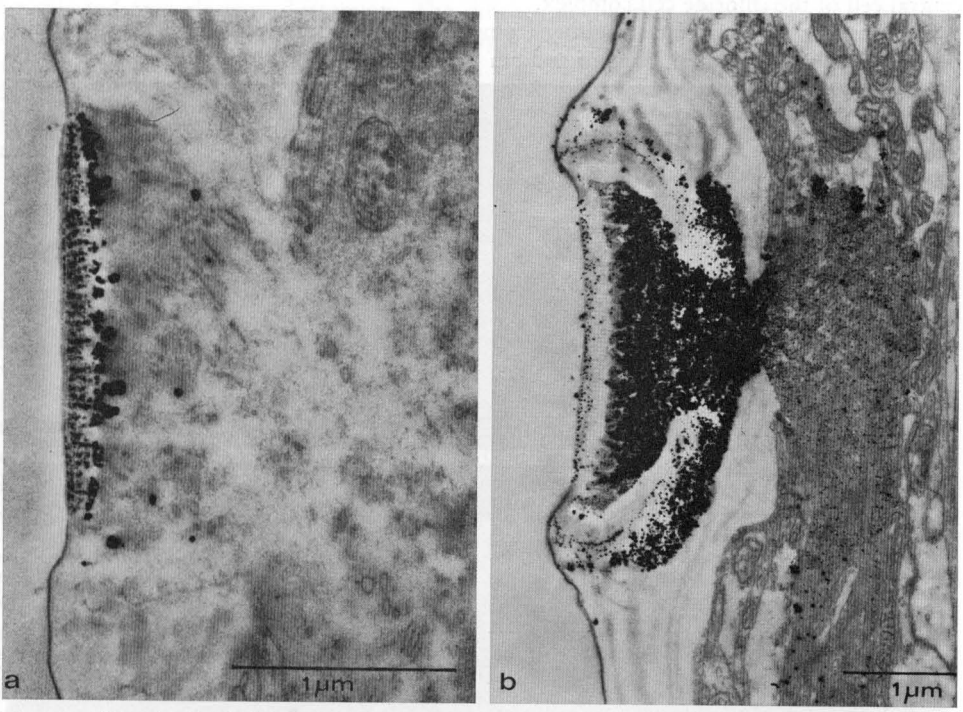

Fig. 6 a. Histochemical demonstration of sodium in the chloride cell complex of *Cloeon*. 28,800 ×. – **b.** Histochemical demonstration of chloride in the chloride cell complex of *Cloeon*. 14,400 ×.

Fig. 5 a. Porous plate and apex of the central cell of a chloride cell complex in *Cloeon*. 40,000 ×. – **b.** Chloride cell complex of *Cloeon* tracheal gill fixed during moult. 10,000 ×. – **c.** Chloride cell complex of *Cloeon* tracheal gill sectioned through the peripheral region of the porous plate, thus the apex of the central cell erroneously appears to be isolated within the cuticle. 13,700 ×.

shaped (fig. 4). The surrounding cells normally have a lighter ground cytoplasm than the central cell. They are highly pleomorphic and possess many irregularly shaped cell processes. These processess interdigitate with similar processes from adjacent cells in the complex and sometimes also with processes extending from the central cell. Thus, in contrast to the chloride cells in *Rhithrogena,* the chloride cell complexes in *Cloeon* have an extensive basal labyrinth [27]. The abundant mitochondria of the additional cells are preferably localized within the interdigitated cell processes (fig. 4, 5 b, c).

Histochemical demonstration of sodium and chloride. Dense precipitates resulting from both, the histochemical reactions for sodium and chloride, are localized only at those sites of the tracheal gills, where cloride cells occur. This holds true for *Rhithrogena* as well as for *Cloeon,* which is demonstrated in fig. 6. In *Rhithrogena* not figured herein, dense precipitates are found within the porous plate and within the apical cavity. In *Cloeon,* the antimonate precipitates indicating sodium, are restricted predominantly to the porous plate, where they appear in lines (fig. 6 a). This is presumably due to the location of precipitate within tiny pore channels. Only a sparse amount of the sodium precipitate is localized within the apical cytoplasma of the central cell of the chloride cell complex.

The amount of precipitate resulting from the silver chloride reaction is always much larger (fig. 6 b) than the amount of sodium precipitates. This is probably at least partly

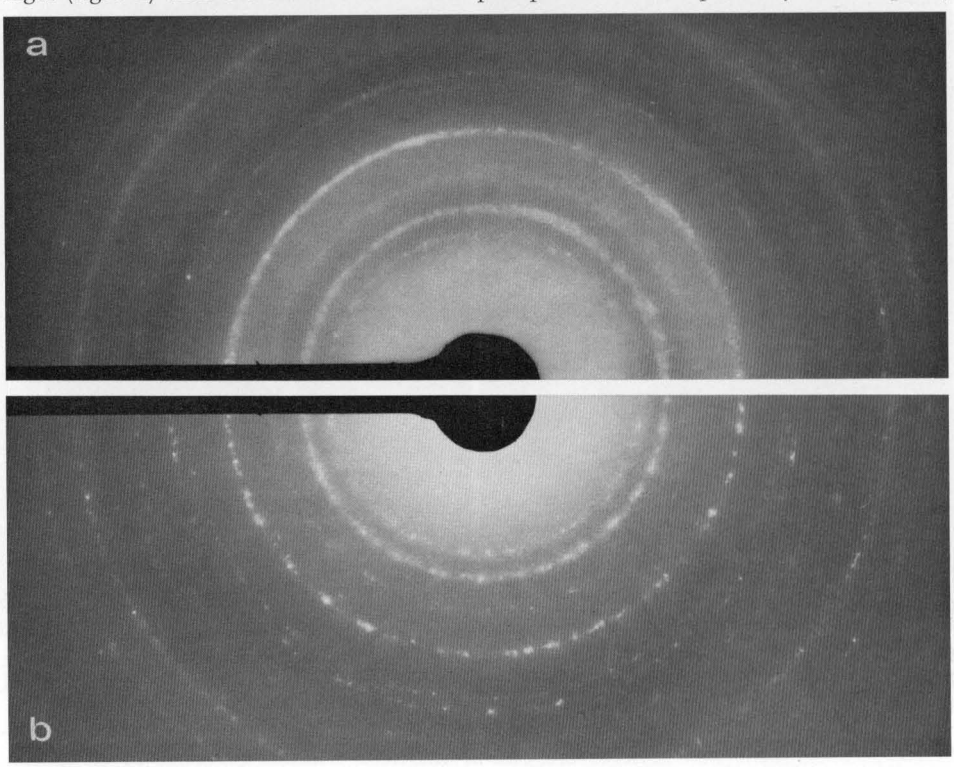

Fig. 7. Selected area electron diffraction diagrams taken at 60 KV from (**a**) the histochemical reaction product in *Cloeon* chloride cell complex and from (**b**) a freshly prepared AgCl suspension, showing identical diffraction patterns.

due to the fact that the kistochemical reaction for demonstrating chloride is more sensitive than that for sodium [15]. Conversely, the larger amount of silver precipitate might also indicate that more chloride is being transported. In other words, other kinds of cations may also be transported and account for the equivalence of chloride. The silver chloride precipitate is generally quite dense in the apical part of the central cell, and less dense in the intermediate and basal part as well as within the porous plate. A zone free of precipitate is often observed around the cell apex, and is followed by a region of the cuticle which has dense precipitates. The latter is thought to be a diffusion artifact resulting from high chloride concentration within the cell apex. Apparently the total amount of chloride at this site is not precipitated immediately, because of the low concentration of silver within the fixative [for detail see 13].

Identification of the silver chloride reaction product. It has already been shown that silver nitrate causes black staining in the anal papillae of mosquito larvae and in the rectal gills of *Libellula* larvae at the light microscopic level [16]. KROGH [16] pointed out that this is due to absorption of Ag^+ from dilute concentrations. In order to ensure that the heavy precipitate in *Cloeon* tracheal gills is really a silver chloride precipitation rather than an absorption of silver from the fixative, the precipitates were identified by selected area electron diffraction. The diffraction diagram obtained from the histochemical reaction product (fig. 7 a) is actually identical with the diagram obtained from freshly prepared silver chloride suspensions (fig. 7 b). As pointed out previously [13], both diagrams indicate the presence of silver chloride and colloid silver, the latter being caused by reduction of silver chloride during the procedure. Thus it may be concluded that originally only silver chloride was present in the chloride cell complexes.

Discussion

The fine structure of the chloride cells within the tracheal gill epithelium of *Rhithrogena* nymphs shows a striking similarity to that of the well-known chloride cells of teleost gills. The chloride cells of the stickleback, for example, when raised in fresh water or adapted to very low external salt concentration have a septate apical cavity [1]. These folds, which are not found in sticklebacks adapted to sea water, are thought to enlarge the absorptive surface of the apical plasma membrane. The structural feature of the apical cavity of *Rhithrogena* chloride cells is very similar, and histochemical localization of sodium and chloride within the apical cavity is the same in both stickleback chloride cells [1, 13] and *Rhithrogena* nymphs' chloride cells.

Contrary to teleost chloride cells, the interior tubular complex of *Rhithrogena* chloride cells opens into the apical cavity, whereas in fish chloride cells this system, which was formerly believed to constitute the smooth endoplasmic reticulum of these cells [22], opens towards the base and into the lateral intercellular spaces [1, 8, 20, 21, 25] as it does in the epithelial cells of the dendritic organ of some marine catfish [18, 19]. The functional significance of this difference is not known and seems difficult to evaluate, because in both marine fish chloride cells, which are believed to excrete salt, and fresh water fish chloride cells, which are believed to absorb salt [24], the tubular channels open towards the base [for possible explanation see 7].

The functional relationship between chloride cells in *Rhithrogena* and the chloride cell complexes in *Cloeon* is indicated structurally by the porous plate and the abundance of mitochondria, and histochemically by the localization of sodium and chloride. The presence of a basal labyrinth makes these cells complexes markedly similar to other ion

transporting epithelia, which are either absorptive [e. g. 5, 6, 33] or excretory [e. g. 4, 14] in function.

From our morphological and histochemical results, we may suggest, however, that these cells of the ephemerid tracheal gills are indeed chloride cells comparable in function to the teleost gills chloride cells. But we cannot decide from these data only, whether their main function is the obsorption or excretion of ions. As the osmotic pressure of the hemolymph in ephemerid nymphs corresponds to about 141 to 148 mM NaCl, the Na^+ and Cl^- concentrations of the hemolymph being 103 and 110–177 meq/l respectively [32], and thus is within the same range as that of the hemolymph of mosquito larvae [16], there must be a similar osmoregulatory situation in these fresh water insect larvae. Therefore, we may conclude that the ephemerid nymphs' chloride cells are involved in osmoregulation and that their main function is to absorb ions such as sodium and chloride from the outside medium.

The porous plate of the ephemerid chloride cells, which of course does not exist in fish, is closely related to the fact that these animals are enveloped within a chitinous exoskeleton probably of low permeability [16]. As indicated by the results of the histochemical sodium localization, the porous plates seem to be specialized cuticular regions of increased permeability. In ephemerid tracheal gills, where salt absorption is apparently restricted to several spots only, the normal cuticle structure does not seem to fulfill the permeability requirements as it does in mosquito larvae anal papillae, which have no porous plates. In the anal papillae, however, the whole epithelium is involved in salt absorption [10], as judged from the fine structure [3, 30]. In addition, when the larvae are raised in low external salt concentrations, the anal papillae increase in size [35], and the apical folds of the epithelium become more elaborate at the same time [30]. Thus as a result, there is an enlargement of the absorptive surface at both the anatomical and cytological level. The apical microvilli and folds of the ephemerid chloride cells may indicate a corresponding adaption at least at the cytological level.

It is still unknown, whether there is any relation between the differences in fine structure of the ephemerid chloride cells and chloride cell complexes and the differences in habitat of the species studied. Furthermore, some differences in the osmoregulatory conditions of these species might also exist, which could affect the differentiation pattern of the chloride cells in these closely related animals. Therefore, the ephemerid chloride cells seem to be a suitable model for further and more detailed investigation of the correlation between structure and function.

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Zusammenfassung

Im Epithel der Tracheenkiemen von Eintagsfliegenlarven wurde ein besonderer Zelltyp beobachtet, der den Chloridzellen der Fischkiemen ähnelt und daher ebenfalls Chloridzelle genannt wurde.

Die Chloridzellen von *Rhithrogena semicoloratta* sind Einzelzellen, die durch folgende Strukturmerkmale gekennzeichnet werden: Ihren Mitochondrienreichtum, ihr apikales Labyrinth, das aus tubulären Zellmembraneinfaltungen besteht, ihre apikale Höhle mit zahlreichen Mikrovilli, sowie durch eine Porenplatte, welche eine örtliche Spezialisierung der Cuticula über der apikalen Höhle darstellt.

Die Chloridzellen von *Cloeon dipterum* sind Zellkomplexe. Der konische Apex der Zentralstelle liegt in einer trichterförmigen Aussparung der Cuticula und stößt direkt an die äußere Porenplatte. Die Hüllzellen der Zentralzelle sind stark miteinander verzahnt, so daß diese Zellkomplexe ein typisches basales Labyrinth aufweisen.

Die histochemische Lokalisation von Natrium und Chlorid zeigt bei *Rhithrogena* eine hohe Konzentration dieser Ionen in der Porenplatte und in der apikalen Höhle an, bei *Cloeon* in der Porenplatte und im Apex der Zentralzelle.

Aus diesen Ergebnissen wird geschlossen, daß die Chloridzellen der Ephemeridenlarven an der Osmoregulation dieser Tiere beteiligt sind und ihre Hauptfunktion wahrscheinlich die Absorption von Electrolyten ist.

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