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Fine Structure and Function of the Abdominal Chloride Epithelia in Caddisfly Larvae

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Summary. The larvae of the caddisfly *Anabolia nervosa* Curt. (Limnephilidae) possess 10 fields on the dorsal and ventral sides of the 3rd to 7th abdominal segments, which were formerly regarded as specialized sites of respiration. The epithelial fine structure and histochemical localization of chloride unequivocally show that the main function of these sites is the transport of electrolytes. They probably participate in osmoregulation by the absorption of salt. Therefore, these specialized areas of the hypodermis are termed chloride epithelia.

Key words: Caddisfly larvae — Abdominal chloride epithelia — Fine structure — Histochemical chloride localization — Electrolyte transport — Osmoregulation.

Introduction

On the abdominal segments of caddisfly larvae belonging to the family Limnephilidae (Trichoptera), circumscribed areas are present that show strong argyrophilia when the animals are placed into dilute silver nitrate solution. This staining reaction was thought to be due to a reduction of silver ions resulting from the activity of respiratory cells in these abdominal areas. Accordingly, the underlying epithelia have been functionally interpreted as skin areas specialized for respiration and termed 'respiration fields' or 'gill plates' (Krawany, 1935). However, evidence will be presented in this paper that the reduction of silver ions at these sites is a photochemical secondary process of a primary histochemical chloride reaction. The fine structural and histochemical results obtained so far clearly demonstrate that the so-called 'gill plates' of these freshwater animals are mainly involved in electrolyte transport. According to their chloride transporting function and in analogy to the ephemerid chloride cells described previously (Wichard and Komnick, 1971b; Komnick, Rhees and Abel, 1972; Wichard, Komnick and Abel, 1972) these specialized areas of the hypodermis are termed chloride epithelia.

Material and Methods

Larvae of *Anabolia nervosa* Curt. (Limnephilidae) were collected from the 'Heiliges Meer', a small lake in Westfalia (Wichard and Beyer, 1972), and kept in small, plant-containing outdoor basins until use. Only larvae of the 5th instar were used in this study.

For fine structural investigation whole animals were immersed in 2% osmium tetroxide containing 0.1 M cacodylate buffer at pH 7.2. Immediately after immersion the abdomen was cut at the anterior and posterior ends and the intestine removed, so that the fixative had access to the abdominal hypodermis from both sides. Subsequently, the specimens were fixed for 2 hours at room temperature and rinsed in buffer. While in buffer, the abdominal segments bearing chloride epithelia were isolated and divided into dorsal and ventral halves, so thaeach tissue block contained one chloride epithelial field. Further treatment included dehydrat

tion in graded alcohols, staining in 0.5% uranyl acetate and 1% phosphotungstic acid (Wohlfarth-Bottermann, 1957), embedding in Araldite (Glauert and Glauert, 1958), thin sectioning on the LKB ultramicrotome, additional staining of some sections with lead citrate (Reynolds, 1963) and examination with the Philips EM 200 electron microscope.

For histochemical demonstration of chloride the larvae were fixed for 2 hours with 2% osmium tetroxide and 1% silver lactate in 0.1 M cacodylate acetic acid buffer at pH 6.0, dissected, treated with 0.1 N nitric acid during dehydration, and processed for electron microscopy (Komnick, 1962; Komnick and Bierther, 1969).

For identification of the silver precipitates selected area electron diffraction was comparatively performed on thin tissue sections and suspension preparations of freshly precipitated silver chloride.

The following topochemical reactions were carried out on the light microscopical level in order to elucidate the basis of argyrophilia described by Krawany (1935):

1. a) Fixation with 0.1 N $AgNO_3$ in 1 N HNO_3 for 1 hour.
   b) Washing in 1 N HNO_3 for 15 minutes.
   c) Rinsing in distilled water for 15 minutes and dissection of the abdominal segments.
   d) Exposure to bright light or treatment with 10% alkaline formaldehyde.
2. a) Fixation with 2% osmium tetroxide and 1% silver lactate in 0.1 M cacodylate-acetic acid buffer at pH 6.0 for 2 hours.
   b) Washing in 1 N or 10 N HNO_3 for 15 minutes.
   c) Rinsing in distilled water for 15 minutes and dissection of the abdominal segments.
3. a–c) The same as under 2.
   d) Treatment with 10% $(NH_4)_2\ CO_3$ for 2 hours.
4. a) Fixation in 50% ethyl alcohol for 5 minutes.
   b) Dissection of the abdominal segments and washing in 10 changes of distilled water for a total of 1 hour.
   c) Treatment with 0.1 N $AgNO_3$ in 1 N HNO_3 for 30 minutes.
   d) Washing in 1 N HNO_3 for 15 minutes.
5. The same procedure as listed under 4., but between step b and c an additional 1 hour treatment with 10 mM NaCl was included.

All preparations were finally rinsed in distilled water, dehydrated in graded alcohols, and photographed with the Zeiss Tessovar using reflecting light. Preparations processed according to procedures 4. and 5. were also embedded for electron microscopy.

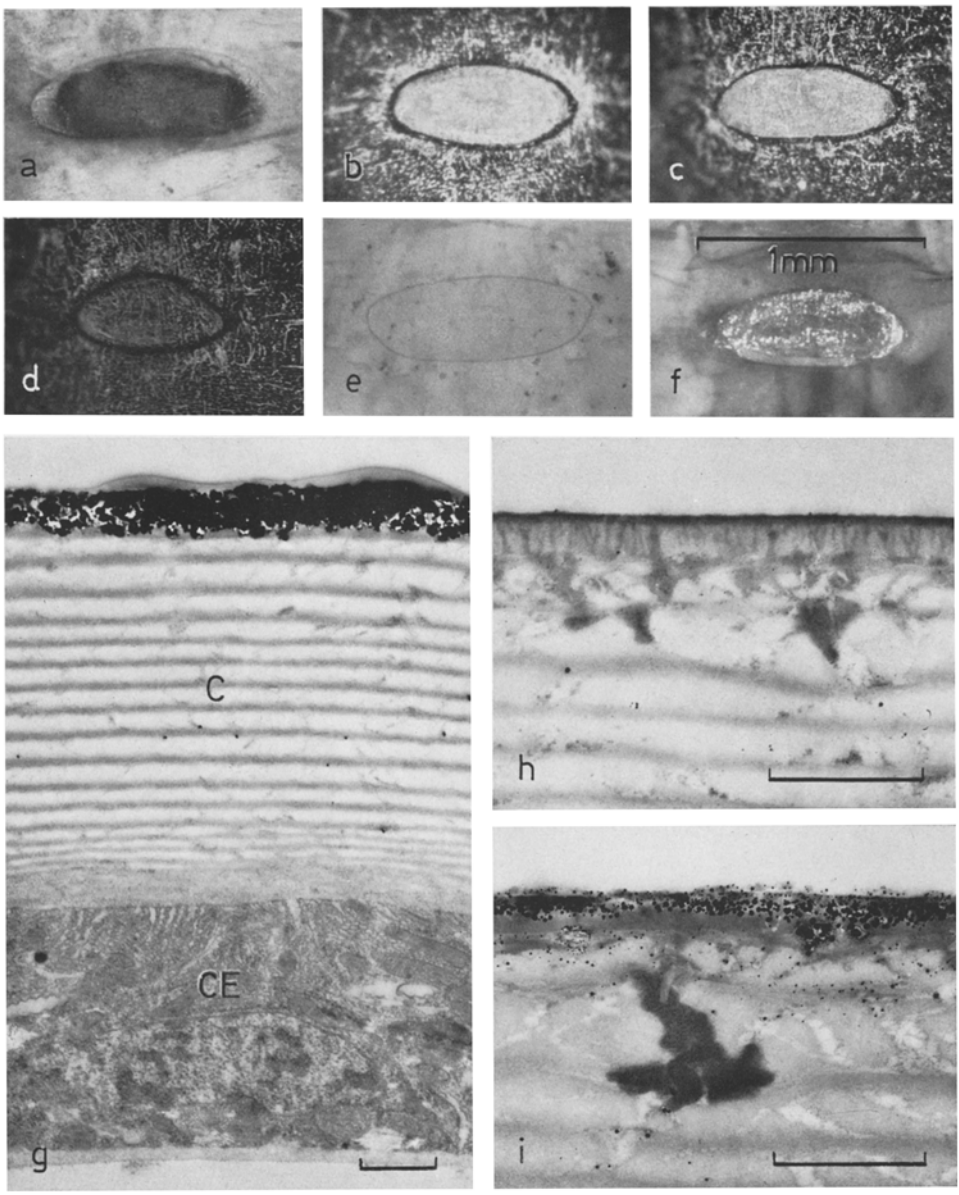
Observations

The larvae of *Anabolia nervosa* possess 10 fields of chloride epithelia. These are located on the dorsal and ventral sides of the 3rd to 7th abdominal segments and represent specialized areas of the hypodermis. They are of oval shape (Figs. 1a–f) and of variable sizes. In the 5th instar they measure approximately 0.2 to 1.0 mm in length and 0.1 to 0.4 mm in width.

Evaluation of argyrophilia and histochemical demonstration of chloride.

When the larvae are fixed in acidic silver nitrate, the fields become clearly visible under the dissecting microscope by attaining a white colour, which turns

Fig. 1a—i. Demonstration of chloride within the cuticle lining the abdominal chloride epithelia of larval *Anabolia nervosa*. a Fixation with 0.1 N $AgNO_3$ in 1 N HNO_3 and subsequent reduction of the original white AgCl precipitates to colloidal silver with alkaline formaldehyde. b Fixation with 2% osmium tetroxide and 1% silver lactate results in white AgCl precipitates. c The same fixation as in (b). Additional treatment with 1 N HNO_3 does not dissolve the precipitates. d The same fixation as in (b). Additional treatment with 10% ammonium carbonate dissolves the precipitates. e Removal of the cuticular chloride by washing with distilled water, followed by fixation with 0.1 N $AgNO_3$ in 1 N HNO_3. f After removal of the cuticular chloride with distilled water the preparation was immersed in 10 mM NaCl for 1 hour and subsequently fixed with 0.1 N $AgNO_3$ in 1 N HNO_3. This treatment shows that the cuticle of the chloride



epithelia deprived of chloride again specificly adsorbs chloride from dilute concentration. Reflecting light; ×30. (The white lines and dots surrounding the chloride epithelia appear only in preparations fixed with the osmium/silver solution (Figs. 1 b–d). They are partly due to light reflections. Since they do not dissolve in nitric acid (Fig. 1 c) nor in ammonium carbonate (Fig. 1 d) and do not show up after reduction of silver (Fig. 1 a), they do not result from precipitation or binding of silver). g Electron micrograph of a cross section of the chloride epithelium, showing dense precipitates in the epicuticle. *C* cuticle; *CE* chloride epithelium. ×10000. h Removal of chloride by washing with distilled water prior to fixation with the histochemical chloride reagent. × 20000. i Adsorption of chloride from 10 mM NaCl after chloride removal with distilled water. ×20000. (bars = 1 μ, unless stated otherwise)

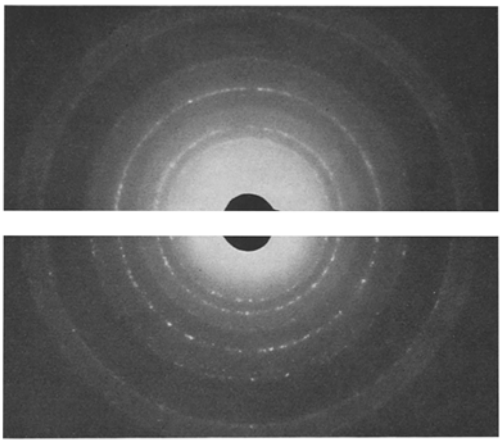


Fig. 2. Selected area electron diffraction diagrams taken from the precipitates in the cuticle of the chloride epithelium (upper half) and from a suspension of freshly prepared silver chloride (lower half)

violet to brownish black, when exposed to bright light or treated with alkaline formaldehyde (Fig. 1a). Fixation with the osmium tetroxide silver lactate mixture also results in white precipitates, which are specifically confined to the oval fields (Fig. 1b). The precipitates do not dissolve in nitric acid (Fig. 1c) even when used in 10 N concentration. Subsequent treatment with ammonium carbonate, however, completely dissolves the precipitates (Fig. 1d). These results unequivocally indicate that the precipitates are silver chloride (Jander and Wendt, 1954). Accordingly, the argyrophilia described by Krawany (1935) is a secondary photochemical reduction of silver, the primary reaction being the precipitation of silver chloride.

In thin sections of specimens fixed in the osmium silver mixture dense precipitates specifically line the cuticle overlying the chloride epithelium (Fig. 1g). They are localized predominantly within the dense layer of the epicuticle, a few being scattered within the procuticle. Selected area electron diffraction diagrams obtained from these histochemical precipitates in thin tissue sections and from suspensions of silver chloride are identical (Fig. 2) and reveal the presence of silver chloride and colloidal silver, the latter resulting from a partial reduction of silver in the electron beam (Komnick and Bierther, 1969). These results clearly show that the external part of the cuticle covering the chloride epithelia contains a large amount of histochemically demonstrable chloride. This chloride can be removed by gentle fixation in 50% alcohol and subsequent washing in distilled water (Figs. 1e and h). However, this treatment does not abolish the ability of the epicuticle to accumulate chloride because after subsequent incubation in 10 mM NaCl, chloride is specifically present again in the cuticle of the chloride epithelia and can be visualized by both light and electron microscopy (Figs. 1f and i). Although the amount of chloride is smaller in comparison to the results of chloride precipitation in the living animal (compare with Figs. 1b and g), the cuticular property is only

quantitatively but not qualitatively affected by alcohol fixation. These observations indicate 1) that the cuticle of the chloride epithelia, unlike the cuticle of the normal hypodermis, is able to adsorb and accumulate chloride from dilute concentration; 2) that this ability is independent of the action of the underlying cells and, therefore, must reside in the structural or chemical differentiation of the epicuticle.

Fine Structure

The chloride epithelia of *Anabolia nervosa* are distinctly thicker than the adjacent normal hypodermis of the abdominal segments (Figs. 3a and b). They measure 5 to 6 μ in thickness, whereas the hypodermis is only 2 to 3 μ thick. A reverse relation is found with regard to the thickness of the overlying cuticles. The cuticle of the chloride epithelium measures 5 to 6 μ across and is of about the same thickness as the epithelium itself, whereas the cuticle lining the adjacent hypodermis measures 7 to 8 μ and distinctly exceeds both the hypodermis itself and the cuticle of the chloride epithelium.

In addition to these dimensional differences there is a clear-cut border between the chloride epithelium and the normal hypodermis (Fig. 4a). Along this border the procuticle is lacking. The epicuticle is at least twice as thick as usual and contains abundant pore canals (Figs. 4a, b). The normal hypodermis and chloride epithelium are also demarcated by a line of larger cells, which are highly pleomorphic and interdigitated along the lateral surfaces. The apical portions of these cells occupy the gap in the procuticle. The apices are very slender; they bear short folds, which interlock with the epicuticle (Figs. 4b, c).

Apart from the different thicknesses no distinctive fine structural differences between the cuticle of the chloride epithelium and the normal hypodermis were found. Pore canals, which may provide transcuticular pathways of solutes (Brück and Komnick, 1971) are present at both sites (Fig. 3). In the dense layer they appear to split into finer canals (Fig. 1h) resembling the wax canals of *Blaberus* (Brück and Stockem, 1972). Irregularly shaped patches of presumably dense layer material are conspicuous within the distal region of the procuticle (Figs. 1h and i).

The cells of the chloride epithelia differ markedly from the normal hypodermis by their high content of mitochondria (compare Fig. 3a with b). The abundance of mitochondria in these cells is particularly apparent in tangential sections (Fig. 6). At the same time, tangential sections reveal that the mitochondria seem to be concentrated in the apical portion of the cells. This preferential location is normally not recognized in cross sections of the epithelium (Figs. 3a, 5). The mitochondria are densely packed with cristae, which is a characteristic feature of the mitochondria in cells with high energy metabolism. Furthermore, their matrix contains intramitochondrial granules (Fig. 5), which have been shown to accumulate divalent cations (Peachy, 1964).

Another feature of the cells are the infoldings of the apical plasma membrane. These are also observed in the normal hypodermis (Fig. 3b). However, in the chloride epithelium they are more elaborate (Figs. 3a, 5). While in cross sections the infoldings are more or less perpendicularly oriented to the cuticle and fairly straight (Fig. 5), they show a very irregular, tortuous course in tangential sections (Fig. 6). Furthermore, when cut directly underneath the cuticle, round profiles are

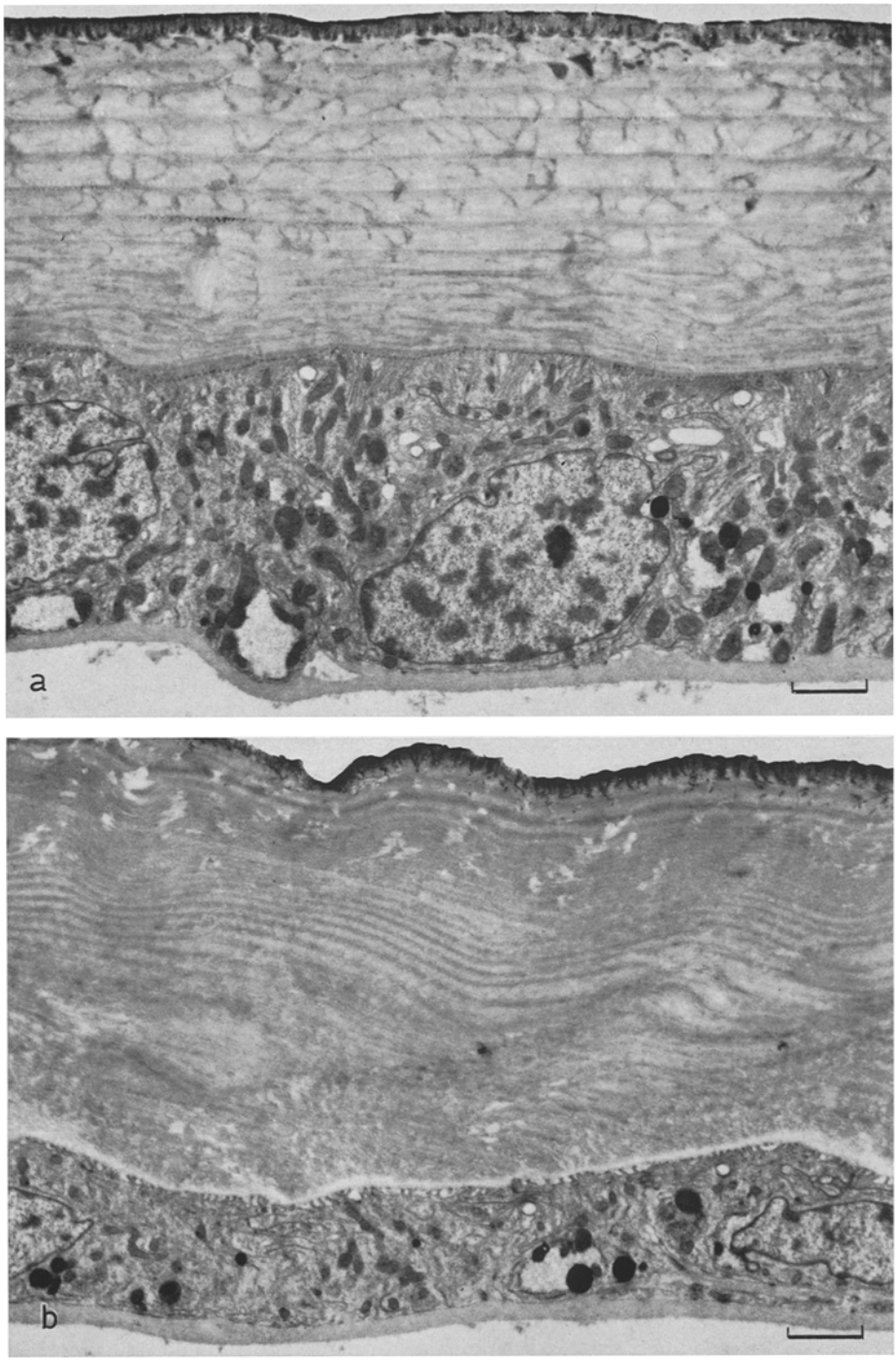


Fig. 3. a Cross section through the abdominal chloride epithelium in *Anabolia nervosa*. b Cross section through the adjacent hypodermis. ×8000

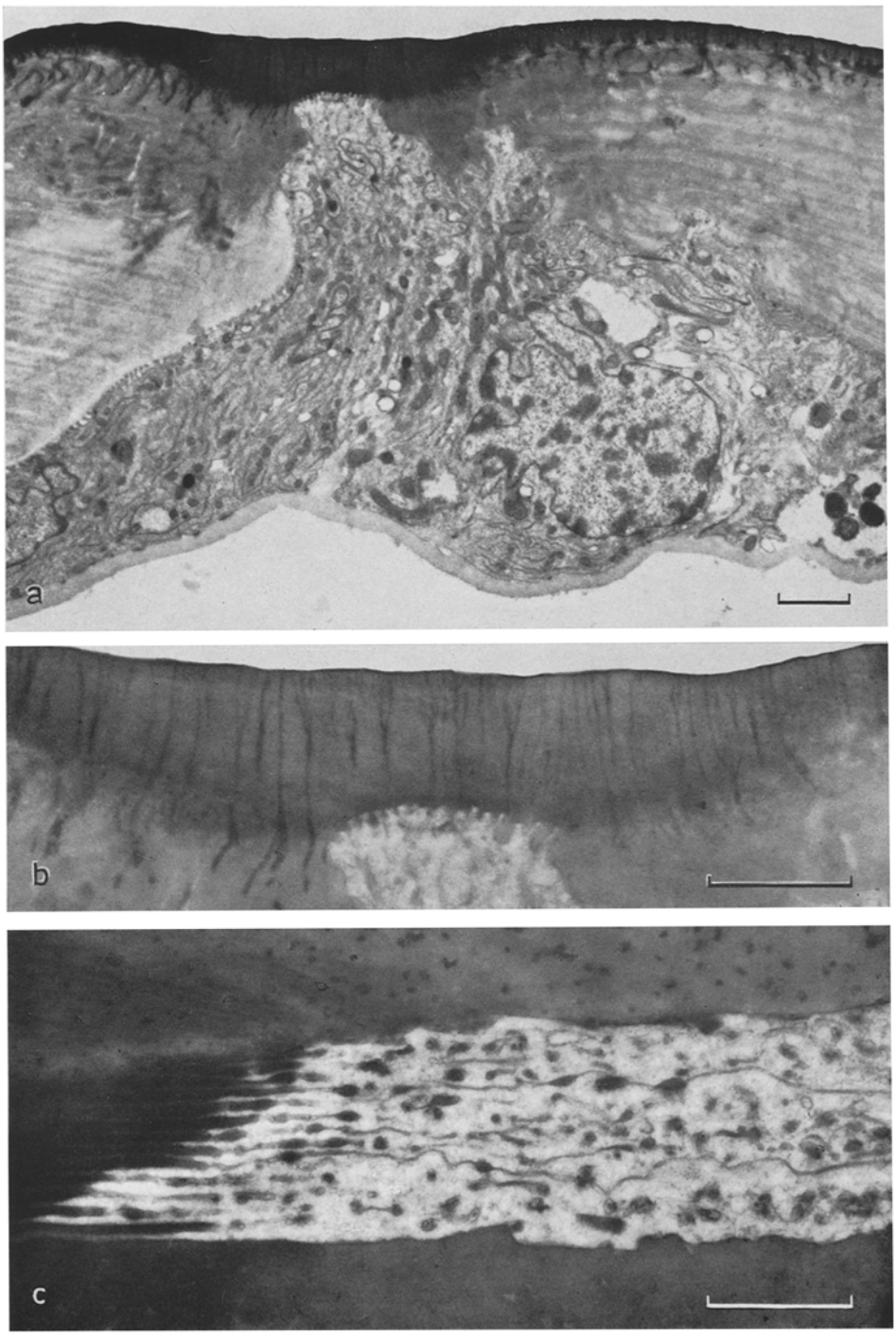


Fig. 4. a Demarcating cells between the chloride epithelium (right hand side) and normal hypodermis (left hand side). ×8000. b and c Apices of the demarcating cells in cross (b) and tangential (c) section. ×20000

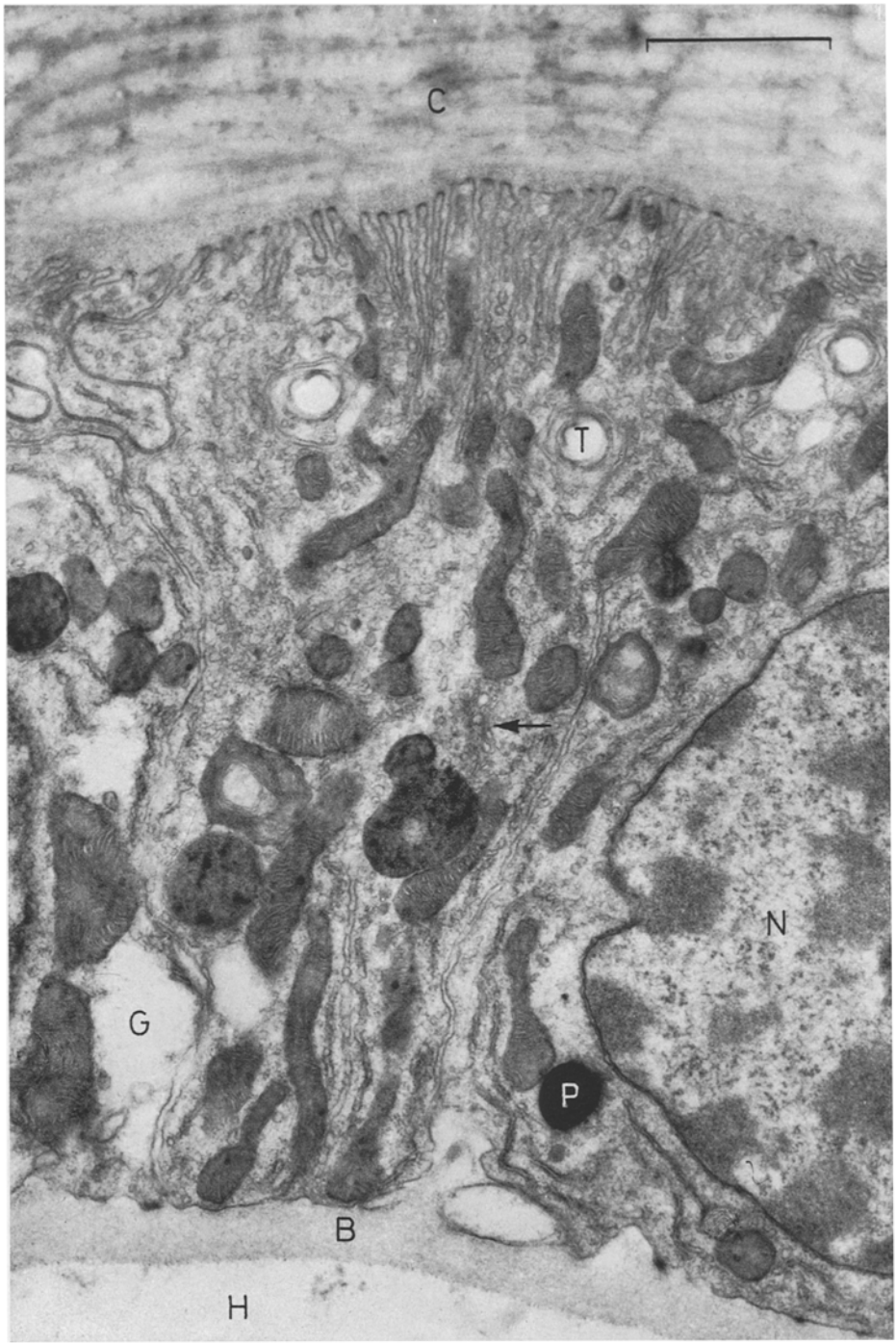


Fig. 5. Cross section of the chloride epithelium in *Anabolia nervosa*. *B* basal lamina; *C* cuticle; *H* hemocoel; *G* glycogen; *p* pigment granule; *T* tracheole; *N* nucleus. The arrow points to a dictyosome. ×25000

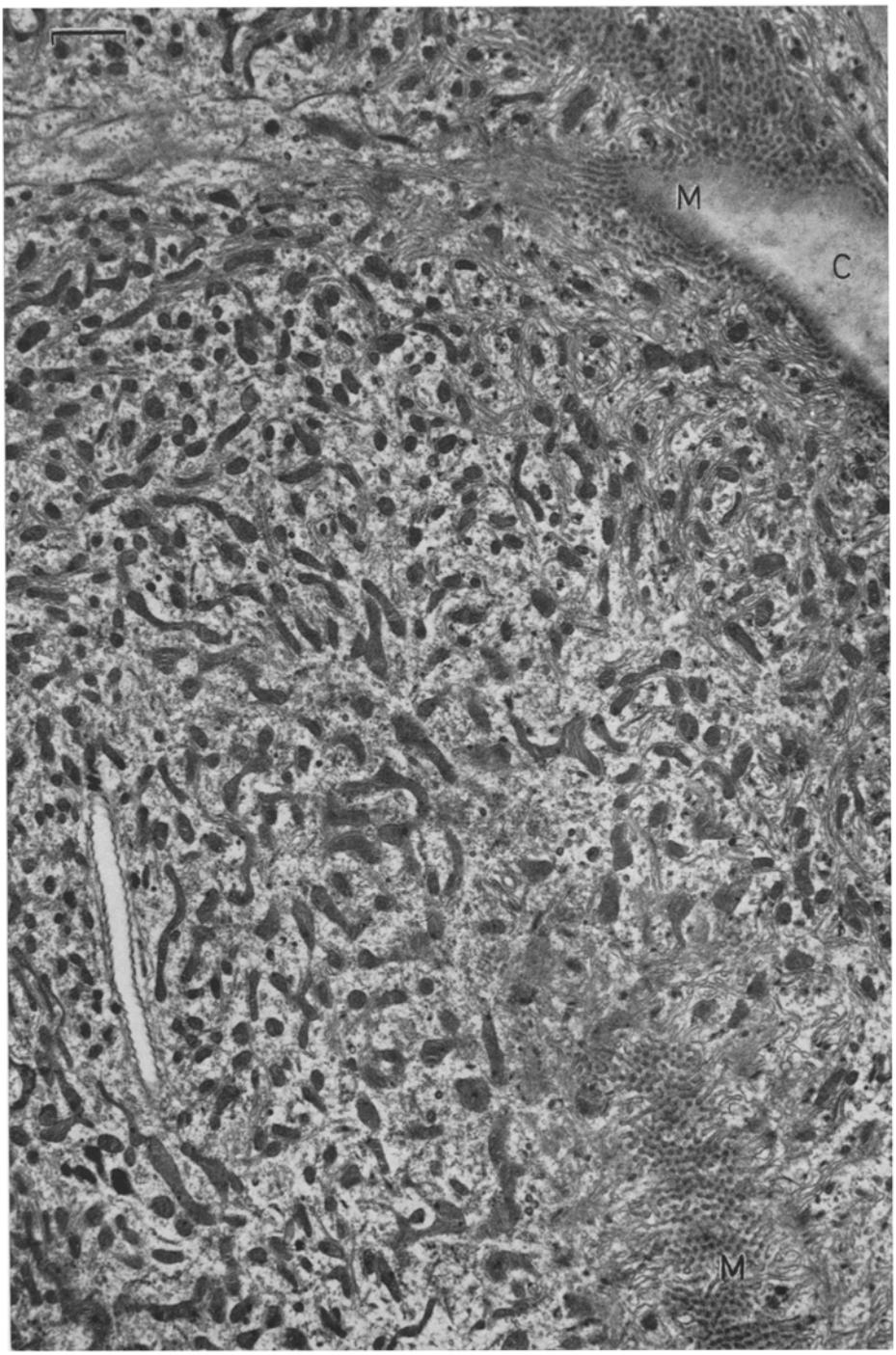


Fig. 6. Tangential section through the apical region of the chloride epithelium. *C* cuticle; *M* microvillous tips of the apical folds. ×10000

visible, which indicate that the most distal parts of the cytoplasmic extentions are more regularly shaped in the form of microvilli (Fig. 6). The microvillous tips facing the cuticle normally appear very electron dense (Fig. 5). In cross sections the apical cytoplasm shows numerous vesicles (Fig. 5), which are occasionally arranged in rows and presumably represent section profiles of the tortuous infoldings (Tormey, 1963). Mitochondria often penetrate into the zone of apical infoldings (Fig. 5) and are found in close association with the infolded plasma membrane (Fig. 6).

Infoldings of the plasma membrane also occur at the basal side; they are, however, less numerous than at the apical side (Fig. 5). Extensions of the basal lamina, which faces the hemocoel, occasionally invade the basal infoldings. Along the lateral sides the cells are irregularly interdigitated, so that the lateral plasma membranes also show a winding course. In the apical region the cells are linked together by desmosomal and septate junctions (Fig. 5).

The basal region of the cells often shows large patches of glycogen, rough endoplasmic reticulum, pigment granules and large dense bodies, which presumably represent mitochondria in various stages of transformation (Figs. 3a, 5). The Golgi apparatus is relatively small and located in the intermediate region (Fig. 5). The nuclei also occupy the basal and middle region of the cells (Figs. 3a, 5). They often show deep indentations.

At places, tracheoles are found in the apical and also deeper regions of the chloride epithelium (Figs. 3a, 5, 6). Their distribution and frequency within the chloride epithelia is similar to those observed in the normal hypodermis (Fig. 3b).

Discussion

The predominant fine structural features of the chloride epithelia in *Anabolia nervosa* are the abundance of mitochondria and the folding of the plasma membrane in conjunction with extracellular channels. Both are common features of electrolyte transporting epithelia. In particular, the fine architecture of the chloride epithelia very closely resembles the epithelial fine structure of the anal papillae and organs in *Diptera* and *Trichoptera* larvae (Copeland, 1964; Sohal and Copeland, 1966; Eichelberg, Wessing and Polenz, 1973; Nüske and Wichard, 1971, 1972). The fine structure of the epithelium and the accumulation of chloride in the overlying cuticle leave no doubt about the conclusion that the main function of the chloride epithelia is the transepithelial solute transport. However, neither the fine structure nor the chloride accumulation allow any conclusion on the main transport direction i.e. whether there is an excretion or absorption. Nevertheless, we favor the assumption that the chloride epithelia perform an absorptive function and are involved in the osmoregulation of these aquatic animals, since the main problem in the osmoregulation of fresh water animals is to handle the excess of water. Many fresh water animals have been shown to possess specialized cells or epithelia at various sites of the body surface, which are able to actively absorb electrolytes from the external medium in order to compensate for the loss of electrolytes by diffusion and fluid excretion through the excretory organs e.g. frog epidermis (Krogh, 1939), anal papillae of *Diptera* larvae (Koch, 1938), chloride cells of fish (Maetz, 1971) and mayfly larvae (Komnick, Rhees and Abel, 1972). In

caddisfly larvae so far, electrolyte transporting epithelia have been found in the anal papillae of *Philopotamidae* and *Glossosomatidae* (Nüske and Wichard, 1971, 1972). Since in these families abdominal chloride epithelia are not present, and since *Limnephilidae* and also *Goeridae*, which possess abdominal chloride epithelia, are lacking anal papillae, the *Trichoptera* larvae apparently have developed at least two different sites for electrolyte absorption.

The accumulation of chloride in the epicuticle, which in principle is independent of the transport activity of the living cells, suggests that the epicuticle apparently possesses an ion adsorption mechanism similar to the chloride cells in mayfly larvae (Komnick, Rhees and Abel, 1972). This mechanism is favorably located in close contact with the external solution but relatively distant from the surface of the transporting cells. The scarcity of silver chloride precipitates in the procuticle and epithelium might be explained by the assumption that the relatively thick cuticle constitutes a barrier for the quick penetration of the fixative or the transepithelial transport process does not include further steps of local chloride concentration.

The results of this investigation demonstrate that the hypothesis that these abdominal areas represent specialized fields for respiration must be abandoned. The tracheoles present in the chloride epithelium could be nessessary for oxygen supply of the mitochondria-rich cells or they are involved in integument respiration like those found in the normal hypodermis. They show in no way, however, the regular distribution which is characteristic of hypodermal sites specialized for respiration (Wichard and Komnick, 1971a). Therefore, the respiratory activity of the chloride epithelia is certainly low and does not exceed the normal respiratory activity of the integument of these animals. In addition, the larvae of *Anabolia nervosa* possess numerous tracheal gills ($\overline{X} = 114$, 8 ± 5, 5) in the 5th instar, which do show the typical arrangement of tracheoles in respiratory organs of these aquatic insects (Wichard, unpublished).

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References

Brück, E., Komnick, H.: Histochemischer Nachweis der Penetrationswege von Salzlösungen durch die isolierte Insektencuticula. J. Insect. Physiol. **17**, 2027–2034 (1971).

Brück, E., Stockem, W.: Morphologische Untersuchungen an der Cuticula von Insekten. I. Die Feinstruktur der larvalen Cuticula von *Blaberus trapezoideus* Burm. Z. Zellforsch. **132**, 403–416 (1972).

Copeland, E.: A mitochondrial pump in the cells of the anal papillae of mosquito larvae. J. Cell Biol. **23**, 253–263 (1964).

Eichelberg, D., Wessing, A., Polenz, A.: Struktur und Funktion der larvalen Analorgane von *Drosophila*. Cytobiologie (im Druck).

Glauert, A.M., Glauert, R.H.: Araldite as an embedding medium for electron microscopy. J. biophys. biochem. Cytol. **4**, 191 (1958).

Jander, G., Wendt, H.: Lehrbuch der analytischen und präparativen anorganischen Chemie. Stuttgart: S. Hirzel 1954.

Koch, H.J.: The absorption of chloride ions by the anal papillae of Diptera larvae. J. exp. Biol. **15**, 152–160 (1938).

Komnick, H.: Elektronenmikroskopische Lokalisation von Na^+ und Cl^- in Zellen und Geweben. Protoplasma **55**, 414–418 (1962).

Komnick, H., Bierther, M.: Zur histochemischen Ionenlokalisation mit Hilfe der Elektronenmikroskopie unter besonderer Berücksichtigung der Chloridreaktion. Histochemie **18**, 337–362 (1969).

Komnick, H., Rhees, R.W., Abel, J.H.: The function of ephemerid chloride cells. Histochemical, autoradiographic and physiological studies with radioactive chloride on *Callibaetis*. Cytobiologie **5**, 65–82 (1972).

Krawany, H.: Trichopterenstudien. X. Untersuchungen über die Atmungsorgane der Larven. Int. Revue ges. Hydrob. u. Hydrogr. **32**, 241–264 (1935).

Krogh, A.: Osmotic regulation in aquatic animals. Cambridge: Cambridge University Press 1939.

Maetz, J.: Fish gills: Mechanisms of salt transfer in fresh and sea water. Phil. Trans. B **262**, 209–249 (1971).

Nüske, H., Wichard, W.: Die Analpapillen der Köcherfliegenlarven I. Feinstruktur und histochemischer Nachweis von Natrium und Chlorid bei *Philopotamus montanus* Donov. Cytobiologie **4**, 480–486 (1971).

Nüske, H., Wichard, W.: Die Analpapillen der Köcherfliegenlarven. II. Feinstruktur des Ionen-transportierenden und respiratorischen Epithels bei Glossosomatiden. Cytobiologie **6**, 243–249 (1972).

Peachy, L.D.: Electron microscopic observation on the accumulation of divalent cations in intramitochondrial granules. J. Cell Biol. **20**, 95–109 (1964).

Reynolds, E.S.: The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. J. Cell Biol. **17**, 208 (1963).

Sohal, R.S., Copeland, E.: Ultrastructural variations in the anal papillae of *Aedes aegypti* (L.) at different environmental salinities. J. Insect. Physiol. **12**, 429–439 (1966).

Tormey, J. McD.: Fine structure of the ciliary epithelium of the rabbit, with particular reference to "infolded membranes", "vesicles" and the effects of diamox. J. Cell Biol. **17**, 641–659 (1963).

Wichard, W., Beyer, H.: Köcherfliegen *(Trichoptera)* im NSG Heiliges Meer in Westfalen. Decheniana **125**, 43–48 (1972).

Wichard, W., Komnick, H.: Zur Feinstruktur der Tracheenkiemen von *Glyphotaelius pellucidus* Retz. *(Insecta, Trichoptera)*. Cytobiologie **3**, 106–110 (1971a).

Wichard, W., Komnick, H.: Electron microscopical and histochemical evidence of chloride cells in tracheal gills of mayfly larvae. Cytobiologie **3**, 215–228 (1971b).

Wichard, W., Komnick, H., Abel, J.H.: Typology of ephemerid chloride cells. Z. Zellforsch. **132**, 533–551 (1972).

Wohlfarth-Bottermann, K.E.: Die Kontrastierung tierischer Zellen und Gewebe im Rahmen ihrer elektronenmikroskopischen Untersuchung an ultradünnen Schnitten. Naturwissenschaften **44**, 287–288 (1957).

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