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EFFECT OF DIFFERENT SALINITIES ON THE CONIFORM CHLORIDE CELLS OF MAYFLY LARVAE

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Abstract—The larvae of *Callibaetis coloradensis* can tolerate a fairly wide range of salinities at hypotonic concentrations. However, they are more sensitive to increasing than to decreasing salt concentrations. Exposure to isotonic concentration results in profound degenerations of the chloride cells within 1 day. Long-term adaptation to diluted fresh water causes a significant increase in the number of chloride cells, whereas the gradual concentration of fresh water to finally 120 mM sodium chloride within a period of 15 days leads to approximately 50 per cent mortality and significantly reduces the number of chloride cells in the survivors. The same reciprocal relation between the number of chloride cells and the external salinity was found in larvae of *C. floridans* collected from fresh- and brackish-water habitats. These results suggest that the adaptive behaviour of the chloride cells is correlated with the osmoregulatory situation and enables these animals to live in habitats of different salinities.

INTRODUCTION

THE LARVAE of *Callibaetis* (Baetidae) possess coniform chloride cells in their integument, which absorb electrolytes from the external solution and participate in osmoregulation (KOMNICK *et al.*, 1972; WICHARD *et al.*, 1972). Since mayfly larvae normally live in fresh water, which is highly hypo-osmotic in respect to the haemolymph (SUTCLIFFE, 1962), salt uptake through the chloride cells at the body surface probably compensates for the loss of salt resulting from diffusion and fluid excretion. If this interpretation is correct, variation in the external salinity should influence the absorption of salt by the chloride cells. An increase in the external salinity decreases the osmotic gradient thereby reducing salt efflux and water influx. The reduction of osmotic water influx further saves internal salt by reducing the necessity for fluid excretion. Provided that there are no major adaptations of the haemolymph tonicity by inorganic or organic components, an increase in the external salinity decreases the need for salt absorption in osmoregulation and vice versa.

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Since in the case of the excretory salt gland of marine birds (e.g. SCHMIDT-NIELSEN and KIM, 1964; STAALAND, 1967; KOMNICK and KNIPRATH, 1970; ENSOR and PHILLIPS, 1972) and absorptive anal papillae of aquatic insect larvae (WIGGLESWORTH, 1938; SOHAL and COPELAND, 1966) these ecological factors influence the morphology, we were interested to know whether similar correlations exist between the external salinity and the chloride cells of mayfly larvae under experimental and natural conditions.

MATERIALS AND METHODS

The larvae of two North American mayfly species were used in this investigation. The adaptation experiments described below were carried out on larvae of *Callibaetis coloradensis*, which were collected from a small fresh-water pond near Fort Collins, Colorado. For comparison with natural conditions larvae of the closely related species *C. floridans* were used, which is one of the few mayfly species, whose larvae are able to live in both fresh- and brackish-water habitats (ILLIES, 1968). *C. floridans* was collected from a small fresh-water creek in Chaires near Tallahassee, Florida, and from brackish-water ponds in the St. Marks National Wildlife Refuge, Florida. Samples of the fresh and brackish water contained 0·5 and 26 mmoles NaCl/l. respectively.

Adaptation experiments

These experiments were carried out on larvae of *C. coloradensis* in aerated aquaria with green algae added as food. Natural fresh water containing 54 mg/l. total dissolved solids and 0·12 m-moles NaCl/l. was used as a standard, and the larvae maintained therein were taken as control. Short- and long-term adaptation experiments were run with both diluted and concentrated fresh water. Diluted fresh water was made by diluting natural fresh water with distilled water in the proportion of 1 : 100 or 1 : 1000; concentrated fresh water was obtained by adding various amounts of sodium chloride.

For short-term experiments, 10 larvae were exposed to each diluted (1 : 100), normal, and concentrated (150 mM NaCl) fresh water for 3, 6, 12, and 24 hr.

In the final long-term experiment, which was run for 15 days at room temperature, 100 larvae were transferred into each 3 l. of diluted (1 : 1000), normal, and concentrated fresh water (50 m-moles NaCl added per l.). In order to reduce the possibility of uncontrolled variation in the salt concentration, the diluted fresh water was changed every day and before addition the algae were rinsed in distilled water. The normal fresh water was changed every 3 days. Furthermore, both the aquaria containing diluted and normal fresh water were loosely covered with a glass sheet to reduce evaporation. On the other hand, the aquarium containing concentrated fresh water was left uncovered for further, gradual salt concentration by water evaporation. At the end of the experiment the remaining volume of fluid was measured and the NaCl concentration calculated at 120 mM from the original concentration and volume. The survivors were counted and processed for electron microscopy and counting of chloride cells.

Electron microscopy

The larvae were fixed with 2% osmium tetroxide in 0·1 M cacodylate buffer at pH 7·2 for 2 hr and subsequently washed in buffer. Then the tracheal gills were removed, dehydrated in graded alcohols, stained *en bloc* with uranyl acetate and phosphotungstic acid (WOHLFARTH-BOTTERMANN, 1957), and embedded in styrene–methacrylate (KUSHIDA, 1961).

Cell counts

For chloride precipitation to visualize the chloride cells (KOMNICK and ABEL, 1971) the larvae were fixed with 2% osmium tetroxide containing 1% silver lactate or with 0·1 M silver nitrate in 1 N nitric acid followed by several changes of distilled water. The tracheal gills were removed, dehydrated in graded alcohol, cleared in xylene, and mounted on slides. Using a light microscope at 400× magnification and equipped with an ocular grid, cell counts were made from the nearly uniform tracheal gills of the intermediate abdominal segments.

RESULTS

Tolerance of C. coloradensis *to different salinities*

Preliminary adaptation experiments revealed that the larvae are very sensitive to major changes in salinity. When directly transferred into concentrated fresh water with 100 or 150 m-moles NaCl/l., which is approximately iso-osmotic (SUTCLIFFE, 1962; FORBES and ALLANSON, 1970), all larvae died within a few days. Direct transfer into distilled water also caused a high initial mortality, although a few larvae could tolerate this treatment for several weeks. The mortality observed in the final short- and long-term experiments (Table 1) clearly shows that *C. coloradensis* can better tolerate major dilutions than concentrations of the external salinity and suggests that this species possesses an effective mechanism for hyper-regulation. The high mortality at approximately iso-osmotic situations indicates that there is little, if any, ability of hyporegulation.

TABLE 1—MORTALITY OF *C. coloradensis* LARVAE IN SHORT- AND LONG-TERM ADAPTATION EXPERIMENTS

	Fresh water		
	Diluted 1 : 100	Normal	Concentrated 150 mM
Short term (1 day), $n = 10$	0	0	40%
	Diluted 1 : 1000	Normal	Concentrated 50 to 120 mM
Long term (15 days) $n = 100$	11%	2%	52%

Effect of different salinities on the number of chloride cells

The chloride cells of *Callibaetis* have been shown to absorb sodium chloride from hypotonic external concentrations (KOMNICK *et al.*, 1972). Therefore, they probably play an essential role in hyper-regulation. The amount of salt absorbed per unit of time theoretically can be controlled by regulating the functional activity of the chloride cells and/or their total number per animal. The investigation of the first possibility requires measurements of fluxes in relation to different salinities, which have not yet been performed. However, structural variations of the cells in short-term adaptation experiments may also deliver some indications. For the study of the second possibility long-term adaptation experiments are necessary, which include at least one moult of the larvae. The numerous exuviae observed during the long-term experiments indicated that the larvae had shed, although shedding cannot be assured for each individual.

In one preliminary experiment, which was run for 1 month, a pronounced difference in the number of chloride cells was observed in the tracheal gills of the three experimental groups (Fig. 1). In comparison with the gills of the control larvae maintained in normal fresh water (Fig. 1b) the gills of the larvae kept in concentrated fresh water had a markedly smaller number of chloride cells, which were restricted to the proximal parts of the gills (Fig. 1a), whereas the gills of specimens maintained in diluted fresh water had a distinctly larger number and the chloride cells were distributed almost over the entire gill surface (Fig. 1c).

It is very difficult to count exactly the total number of chloride cells, because these cells occur in the integument of nearly all body parts (KOMNICK and ABEL, 1971). Therefore, only the tracheal gills were used as a test basis. Twenty gills taken from at least 7 to 10 animals were counted from each experimental group. In order to exclude the possibility of major variations, animals of approximately the same body size were selected and only gills of the intermediate abdominal segments were used which are nearly uniform in shape and size. The size range of the gills of *C. coloradensis* was 1·1 to 1·4 mm in length and 0·7 to 0·8 mm in width. The corresponding figures for *C. floridans* are 0·9 to 1·0 mm and 0·6 to 0·7 mm. Despite

TABLE 2—COMPARISON OF THE MEAN NUMBERS OF CHLORIDE CELLS PER GILL IN *Callibaetis* LARVAE ADAPTED TO OR COLLECTED FROM DIFFERENT SALINITIES

	$\bar{x}$	S.D.	*t*-test
C. coloradensis	$n = 20$		$f = 38$
Concentrated fresh water	516, 0	± 147, 3	4, 47***
Normal fresh water	871, 4	± 170, 4	6, 78***
Diluted fresh water	1097, 0	± 160, 9	
C. floridans	$n = 14$		$f = 26$
Brackish water	360, 6	± 127, 8	7, 65***
Fresh water	674, 4	± 84, 8	

*** Highly significant at $P > 0{\cdot}001$.

these precautions a fairly wide range of variation in the number of chloride cells per gill was found in each experimental group. However, when the individual figures obtained are arranged into size classes, they appear to follow normal distribution curves (Fig. 2), which indicate that sufficiently homogenous populations were used. The mean number of chloride cells per gill significantly differs in the three experimental groups (Table 2) and indicates an adaptative behaviour of the chloride cells. Long-term exposure to concentrated fresh water causes a reduction, whereas long-term exposure to diluted fresh water results in an increase in the number of chloride cells.

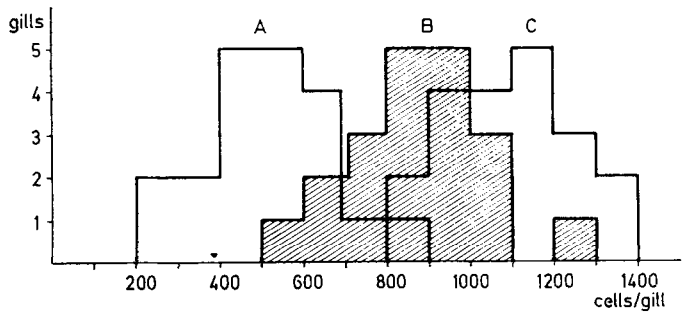

FIG. 2. Distribution of cell counts in the tracheal gills of *C. coloradensis* maintained at different salinities for 15 days. (A) Concentrated fresh water (final concentration 120 mM NaCl). (B) Normal fresh water. (C) Diluted fresh water (1 : 1000).

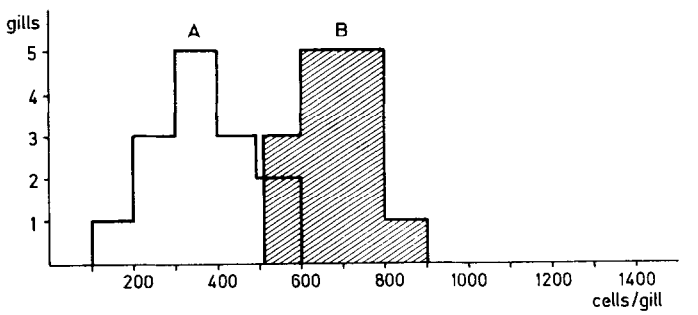

FIG. 3. Distribution of cell counts in the tracheal gills of *C. floridans* collected from brackish and fresh water. (A) Brackish water containing 26 mM NaCl. (B) Fresh water (0·5 mM NaCl).

The same relation between the external salinity and the number of chloride cells was found in *C. floridans* collected from fresh- and brackish-water environments (Fig. 3). Although the differences in the salinities of the natural habitats were much smaller than those of the media used for the adaptation experiments on the related *C. coloradensis*, the mean numbers of chloride cells are significantly different (Table 2). This is probably due to the fact that the larvae had lived in their natural environment for a longer period of time, namely since hatching from the egg.

Effect of different salinities on the fine structure of the chloride cells

The fine structure of the chloride cells and porous plates of *Callibaetis* has been described in detail elsewhere (KOMNICK and ABEL, 1971; KOMNICK and STOCKEM, 1973). The most pronounced fine structural alterations of the chloride cells were observed in the short-term adapted larvae. After exposure to diluted fresh water for 3 hr the apices of the central cells showed numerous deep infoldings of the plasma membrane (Fig. 4a), whereas the apical infoldings were almost completely absent in larvae exposed in concentrated fresh water for 3 hr (Fig. 4b). Prolonged exposure to diluted fresh water for 6, 12, and 24 hr did not cause any additional alterations except that a few chloride cells exhibited slight indications of degeneration. In the concentrated fresh-water experiments, however, various stages of cell degeneration were already detectable in many chloride cells after 3 hr of exposure. After 6 hr of exposure the number of degenerating cells was markedly increased and the degenerative alterations more pronounced (Fig. 4c). After 12 and 24 hr in concentrated fresh water there were scarcely any chloride cells that did not show profound structural degenerations. This finding is in line with the observation that the larvae are unable to survive direct exposure to concentrated fresh water, which is nearly iso-osmotic to the haemolymph, for more than a few days.

In the long-term adaptation experiments, where the larvae were placed into approximately 50 mM sodium chloride, which was gradually concentrated to 120 mM during 15 days, no major differences in the fine structure of the chloride cells of the surviving animals were observed in comparison with the chloride cells of larvae maintained in normal or diluted fresh water for the same period of time.

DISCUSSION

The larvae of *C. coloradensis* are able to tolerate a fairly wide range of variations in the external salinity at hypotonic concentrations. However, they are apparently more sensitive to increasing than to decreasing salt concentrations of their normal fresh-water environment and show a high mortality when the external salinity approaches the osmolarity of the haemolymph. This suggests that they possess a highly efficient mechanism for hyper-regulation, whereas the ability of hypo-regulation, if any, is very poor. It has been shown by FORBES and ALLANSON (1970), that older larvae of *Cloeon crassi* are also able to hyporegulate at concentrations up to twice the osmolarity of the haemolymph. These authors suggested 'that the nymphs normally osmoregulate by drinking the medium and excreting either the excess salts or excess water'. This explanation might be true for hyporegulation. However, drinking for hyper-regulation appears to be unnecessary, since the larvae of Baetidae possess coniform chloride cells (WICHARD and KOMNICK, 1971; WICHARD *et al.*, 1972) which are able to absorb salt from dilute external concentrations. In experiments with radioactive chloride on *C. coloradensis* (KOMNICK *et al.*, 1972) these cells were found only to absorb chloride and did not show any excretory activity after the injection of a hypertonic salt solution. Unless in hyporegulating ephemeropteran species like *Cloeon crassi* the chloride cells are able to reverse their

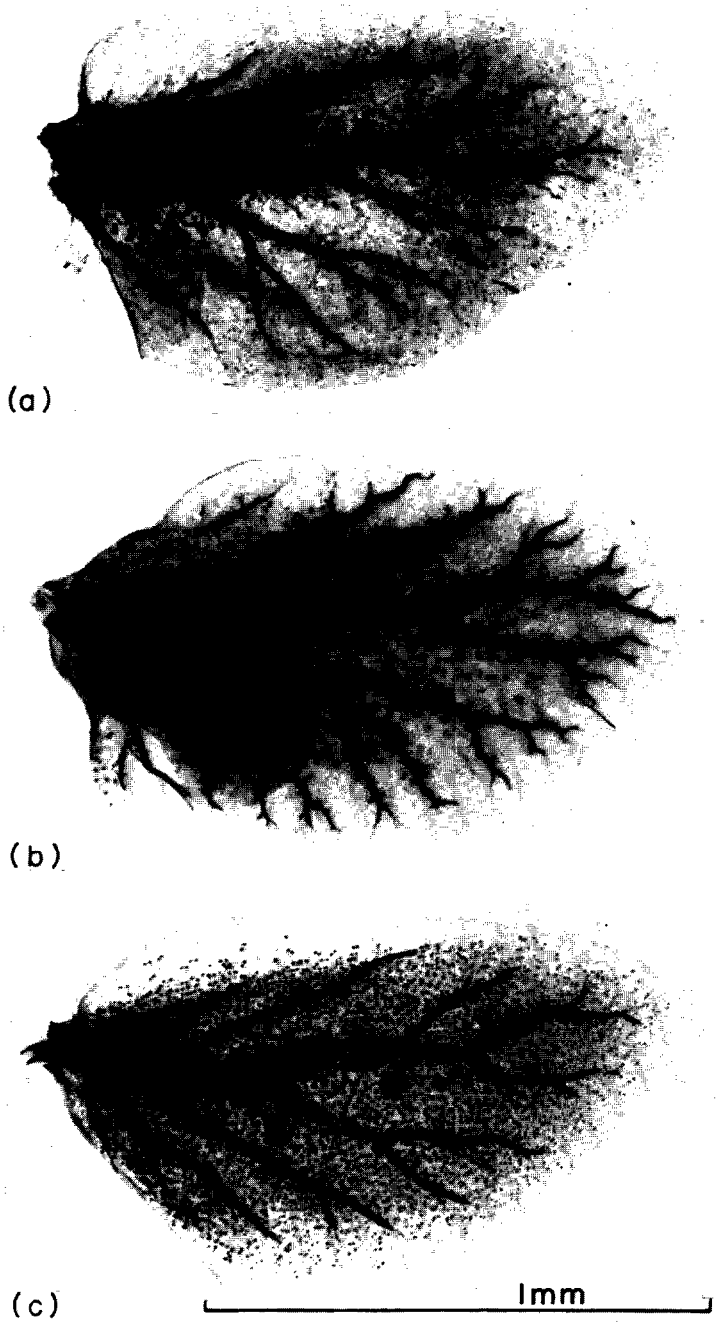

FIG. 1. Tracheal gills of *C. coloradensis* maintained at different salinities (a–c) for 30 days and subsequently fixed in the osmium tetroxide/silver lactate mixture for chloride precipitation. (a) Concentrated fresh water (final concentration 130 mM NaCl). (b) Normal fresh water. (c) Diluted fresh water (1 : 100). Notice the increase in the number of chloride cells at decreasing salinities.

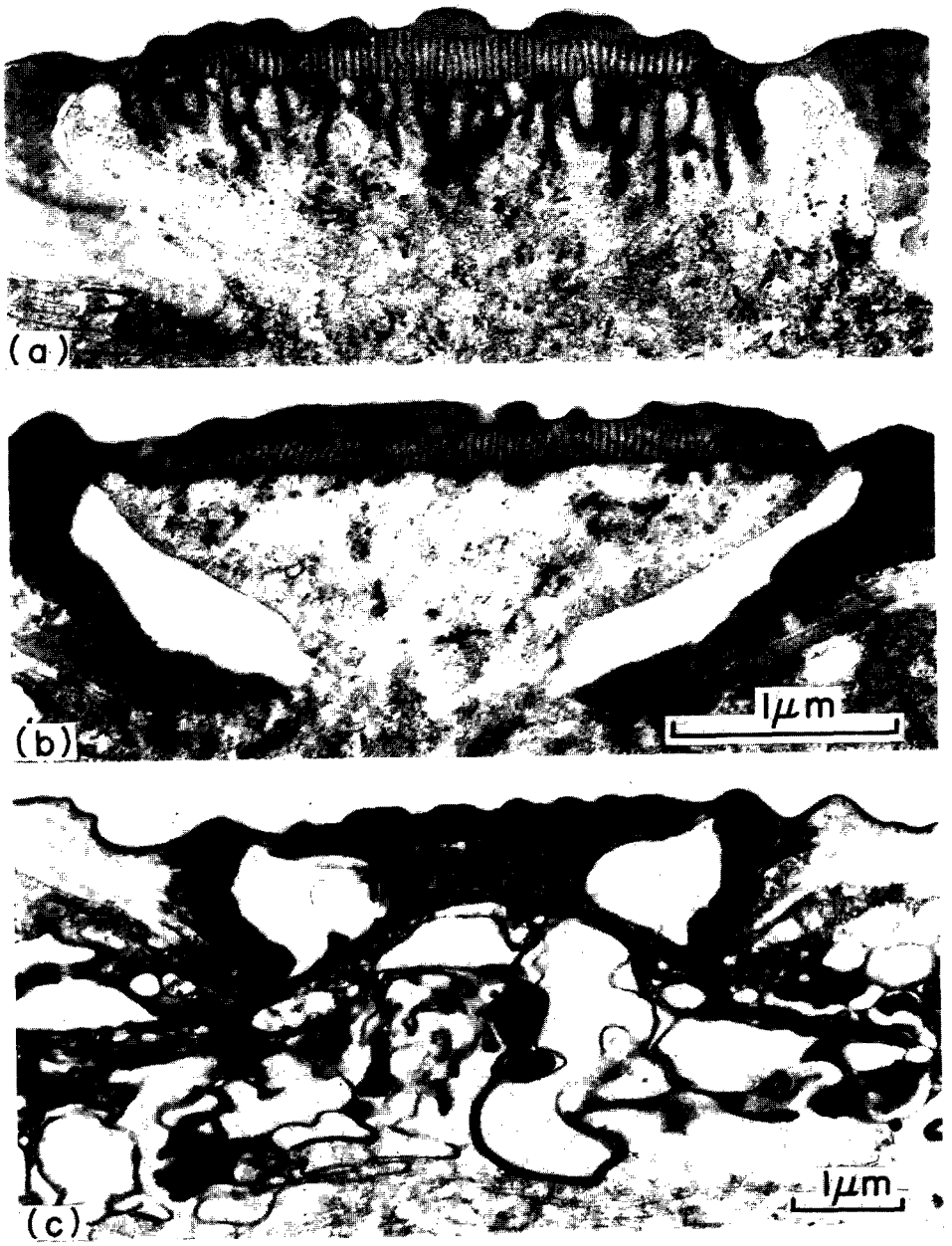

FIG. 4. Fine structural alterations in the chloride cells of *C. coloradensis* exposed to different salinities. (a) Apex of a central cell showing numerous infoldings of the apical plasma membrane after exposure to diluted fresh water (1 : 100) for 3 hr. (b) Apex of a central cell showing a nearly smooth apical plasma membrane after exposure to concentrated fresh water (150 mM NaCl) for 3 hr. (c) A degenerating chloride cell after exposure to concentrated fresh water (150 mM NaCl) for 6 hr.

transport polarity similar to the chloride cells of teleosts, which in fresh-water species absorb and in sea-water species excrete salt, the excretion of the excess salt must occur through the Malpighian tubules by producing hypertonic urine.

The effect of different salinities on the chloride cells of *Callibaetis* as well as the results obtained from the experiments with labelled chloride clearly indicate that these cells play an essential rôle in the hyper-regulation of mayfly larvae. Their increase in number at diluted fresh water and their decrease in number at concentrated fresh water, which were found in two species under experimental and natural conditions, are consistent with the enhanced and reduced requirements of salt absorption for osmoregulation in these media. This adaptive alteration in the number of chloride cells is comparable to the adaptive variation in the size of the absorptive anal papillae (WIGGLESWORTH, 1938) or the excretory salt glands (SCHMIDT-NIELSEN and KIM, 1964) and represents a mechanism, which in the long run and within certain limitations enables the animals to gradually adapt to external changes in the osmoregulatory situation. Such a mechanism appears to be ecologically important in two respects; it enables (1) the species to live in habitats of different salinities and (2) the individuals to survive slow seasonal variations in the salt concentration of small ponds.

Although direct evidence by flux measurements is lacking, the fine structural results of the short-term experiments suggest that the chloride cells are presumably also able to adapt by changing their absorption activity. This possibility may be deduced from the extensive elaboration of the apical infoldings in diluted and their reduction in concentrated fresh-water specimens, since a similar adaptive behaviour of the apical folds was found in the anal papillae of dipteran larvae (SOHAL and COPELAND, 1966). The greater tolerance of the mayfly larvae studied to major dilutions and the minute degenerative effect on the chloride cells seem to reflect that the chloride cells better adapt to sudden dilutions than to sudden concentrations of the fresh-water environment. This adaptive behaviour may also be correlated with ecological factors, since in nature, for example, these animals are faced with sudden dilutions by rainfall.

The conclusion that actually the total number of chloride cells present in the gills and not only the active ones were counted in this study is based on previous results (KOMNICK *et al.*, 1972). It has been shown, that even after ecdysis the central cell apex, which is retained in the shed cuticle, is still able to adsorb chloride independently of the transport activity of the living cell. Therefore, the method of chloride precipitation used for visualization of the chloride cells does not tell anything about the activity of the cells but reveals all chloride cells present. This is also apparent from the results of chloride precipitation in the gills of the larvae from the short-term adaptation experiments. Although thin sections revealed that after exposure to 150 mM NaCl for 24 hr nearly all chloride cells were degenerating, the chloride cells were still visualized by chloride precipitation.

The adaptive variation in the number of chloride cells in relation to different salinities during the long-term adaption experiments can only occur during apolysis because of the formation and removal of the porous plates. As judged from the cell

degenerations observed during short-term exposure to concentrated fresh water, the reduction of the number of chloride cells during long-term adaptation presumably also results from the degeneration of a certain number of chloride cells. The porous plates of the degenerated chloride cells are finally removed with the shed cuticle during moult. On the other hand, the increase in the number of chloride cells during long-term adaption to diluted fresh water is presumably brought about by the differentiation of additional chloride cells. Since the coniform chloride cells of *Callibaetis* are cell complexes (KOMNICK and ABEL, 1971), it seems to be unlikely that new chloride cells arise from simultaneous division of all cells constituting the chloride cell complex. Furthermore, the formation of the porous plate, which is an intricate structural specialization of the cuticle (KOMNICK and STOCKEM, 1973), can probably only take place during the new formation of the cuticle. Therefore, the formation of new chloride cells presumably occurs during apolysis. Since during apolysis a new central cell is apparently invading from the base of the epithelium (KOMNICK and ABEL, 1971), it may well be that entire new chloride cell complexes differentiate during apolysis from normal epithelial cells. However, these assumptions on the degeneration and formation of chloride cells during long-term adaptation experiments require further investigations which include larvae fixed at various intervals of the adaptation period.

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