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The function of ephemerid chloride cells Histochemical, autoradiographic and physiological studies with radioactive chloride on Callibaetis

Die Funktion der Chloridzellen von Eintagsfliegenlarven Histochemische, autoradiographische und physiologische Untersuchungen mit radioaktivem Chlorid an Callibaetis

HANS KOMNICK [1]), REUBEN W. RHEES, and JOHN H. ABEL, JR.
Department of Physiology and Biophysics and Department of Radiology and Radiation Biology, Colorado State University, Fort Collins, Colorado

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Abstract

In experiments using labeled chloride solutions and a combination of the histochemical chloride precipitation technique with autoradiography it was shown that the chloride cells of *Callibaetis* larvae *(Ephemeroptera, Baetidae)* absorb chloride from a hypotonic external salt solution. Experiments on the hemolymph as well as on tracheal gills, fixed in the histochemical chloride reagent after exposure to hypotonic labeled chloride solutions, indicate that the absorption is dependent on both time and concentration and that the chloride cells are involved in osmoregulation.

The apex of the central cell of the chloride cell complex which contains acid mucopolysaccharide and glycoprotein is capable of binding and accumulating sodium and chloride from hypotonic solutions. This property which is independent of the living cell, provides a pool of these electrolytes at the interphase between the external solution and the hemolymph. The significance of these results for the salt absorption from the low concentration of fresh water and for the overall process of transcellular salt transport is discussed.

Introduction

In a previous paper [19] a special cell type was described in the epithelium of tracheal gills of two species of mayfly larvae. The fine structure of these cells, at least in one species, was remarkably similar to the chloride cells in the gills of fish and thus they were termed ephemerid chloride cells. On the basis of the histochemical demonstration

[1]) Present address: Prof. Dr. H. KOMNICK, Institut für Cytologie und Mikromorphologie der Universität, 53 Bonn 1, Gartenstrasse 61 a, Germany. – Supported by the National Science Foundation.

of sodium and chloride in these cells and on the basis of the concentration gradient between the hemolymph and fresh water, it was concluded that the ephemerid chloride cells were involved in osmoregulation and that their main function was probably the absorption of electrolytes.

Since positive histochemical reactions for sodium and chloride indicate only the sites where relatively high concentrations of these ions are present but reveal nothing regarding the dynamics and direction of their transport, experiments using radioactive chloride were performed to determine 1. whether the histochemical localization of chloride in the ephemerid chloride cells reflects an accumulation of this ion from the external media or from the hemolymph and 2. whether there is in fact an absorption of chloride from the external solution into the hemolymph.

Materials and methods

The mayfly larvae used in this study belong to an unidentified species of the genus *Callibaetis (Ephemeroptera, Baetidae)*. They were collected from a small pond near Fort Collins, kept in aerated aquaria and fed green algae. The water for the aquaria was taken from the Cache la Poudre River. It contains 3 mg Na^+/l and 4 mg Cl^-/l and is hereafter referred to as natural water.

1. Histochemistry

Histochemical precipitation of sodium and chloride were performed by the osmium-antimonate or the osmium-silver lactate methods respectively [8, 10]. These technique were applied to gills of living larvae as well as to isolated cuticles collected after moult.

Freshly shed cuticles were also fixed with buffered 3.5 % glutaraldehyde, dehydrated and rehydrated and stained with Hale's colloidal iron or the period acid Schiff reagent [1].

For light microscopical observations whole-mount preparations and 1 µm thick sections of Epon [12] or styrene-methacrylate [11, 16] embedded material were used. Electron microscopy was done on thin sections.

2. Experiments with $^{36}Cl^-$

A 180 mM NaCl stock solution containing $^{36}Cl^-$ and a specific radioactivity of 5.57 µc/ml was diluted to different concentrations for the following experiments. Prior to the experiments *Callibaetis* larvae of approximately 1 cm body length were maintained for 3 days in unlabeled natural water diluted with distilled water in the proportion of 1 : 10.

a. Autoradiography: In one experiment groups of larvae were bathed in a hypotonic labeled chloride solution (15 mM Na ^{36}Cl/l) for 10 or 60 minutes and fixed in the histochemical chloride reagent. In a second experiment several larvae were injected with approximately 0.5 µl of a hypertonic labeled chloride solution (180 mM Na ^{36}Cl/l). Prior to fixation the animals were rinsed for 10 or 60 minutes with running natural water. For this washing procedure especially constructed small plastic vials were used, which ensured rapid exchange of water and thereby removal of labeled chloride excreted by the Malpighian tubules. Therefore, the possibility was excluded that excreted labeled chloride was reabsorbed by

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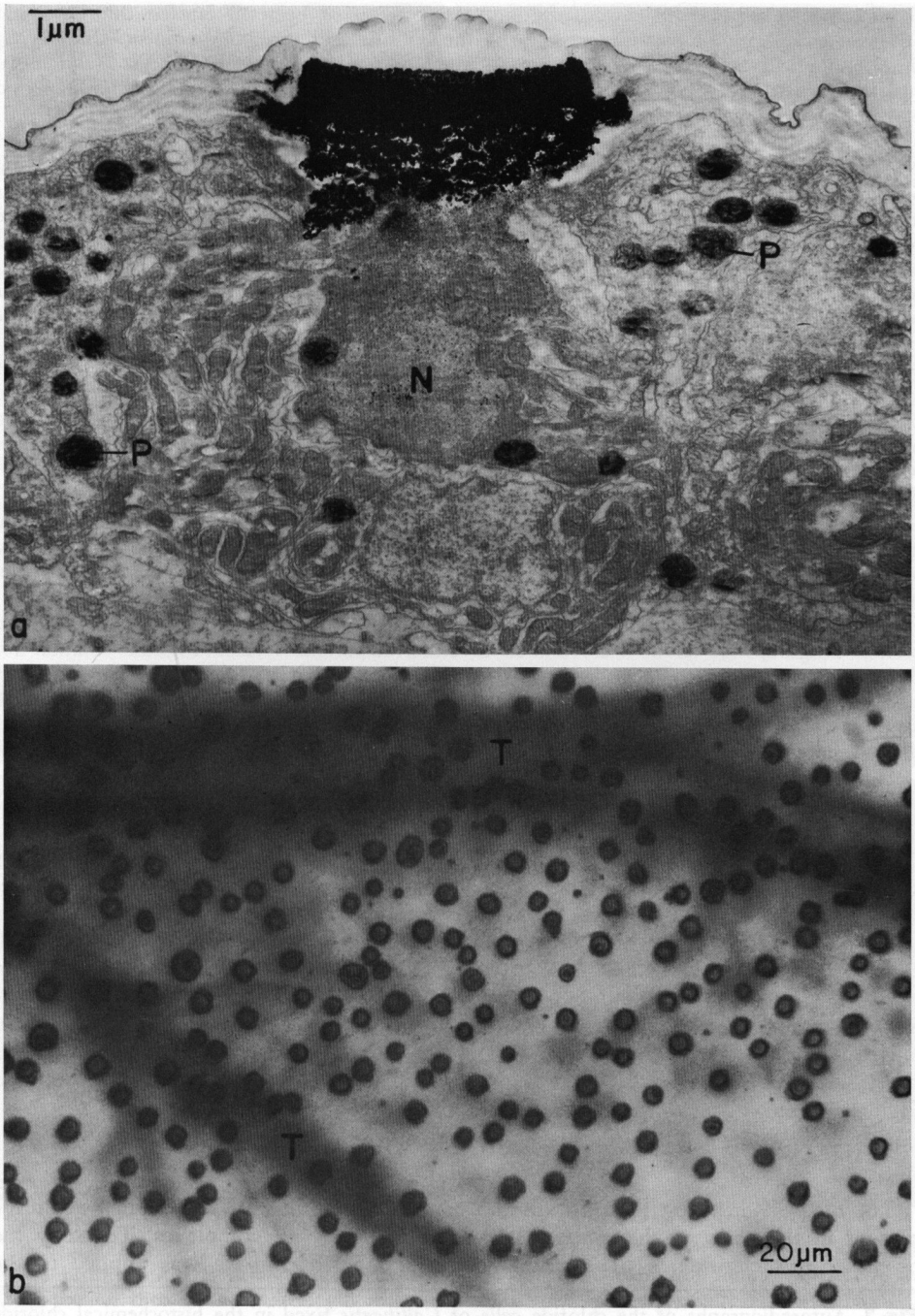
Fig. 1 a. Chloride cell complex within the tracheal gill epithelium of *Callibaetis*, fixed in a OsO_4-silver lactate solution, showing dense AgCl precipitates in the central cell apex. – N Nucleus of the central cell. – P Pigment granules. – 10 000 ×. – **b.** Light micrograph of the surface of a tracheal gill of *Callibaetis* fixed in OsO_4-silver lactate solution for precipitation of chloride. The circular dark dots are AgCl precipitates and indicate the location of chloride cell complexes. – T Trachea. – 500 ×.


1µm
P
N
P
a
T
T
20µm
b

the chloride cells. Without this precaution, reabsorption of excreted labeled chloride could erroneously indicate an excretory function of the chloride cells in this experiment. One micron thick section of the gills embedded in styrene-methacrylate and some isolated abdominal cuticles were coated with Kodak NTB-3 emulsion (dipping technique), exposed for a period of 1 week to 3 months and developed with Dektol.

b. Counting of radioactivity in gills: Groups of larvae were bathed in hypotonic (15 mM Na ^{36}Cl/l) labeled chloride solution for different periods of time (1, 2, 4, 8, 15, 30 and 60 minutes) or in different hypotonic solutions (between 1.5 and 1200 µM Na ^{36}Cl/l) for 1 hour and fixed in the histochemical chloride reagent for just two minutes. This was done to reduce the precipitation of AgCl in deeper portions of the gills. After rinsing in cacodylate-acetic acid buffer the tracheal gills of the 3rd–5th abdominal segments, which are approximately the same size, were removed and further washed in 5 changes of 50 % alcohol. Groups of 10, 20 or 30 gills from the various experiments were placed on filter paper, dried, and the radioactivity was counted with a Low Background Planchet Counter (Nuclear, Chicago) for 10 minutes.

As a control and for background counting one group of larvae was placed in an unlabeled 15 mM NaCl solution for 10 minutes and then treated like the experimental animals. In addition, 0.1 ml of each final washing solution and 0.1 ml of each labeled bathing solution were counted under the same conditions as the gills for comparison of radioactivity and for control of contamination by unprecipitated ^{36}Cl.

c. Morphometric analysis: The gills of the 6th abdominal segments of the experimental animals were also removed and alternatively mounted on glass slides for making cell counts, as well as measurements of AgCl precipitation and gill size, or embedded in styrene-methacrylate for measurements of gill thickness and precipitate thickness in cross-section.

d. Counting of radioactivity in the hemolymph: Similar to the experiments described in section b, groups of larvae were bathed in either hypotonic labeled chloride solution for different periods of time or in different hypotonic concentrations for 1 hour. After a short rinse in natural water 0.75 µl of hemolymph was withdrawn from each animal. The hemolymph samples of each experimental group were placed together on filter paper and counted as described above.

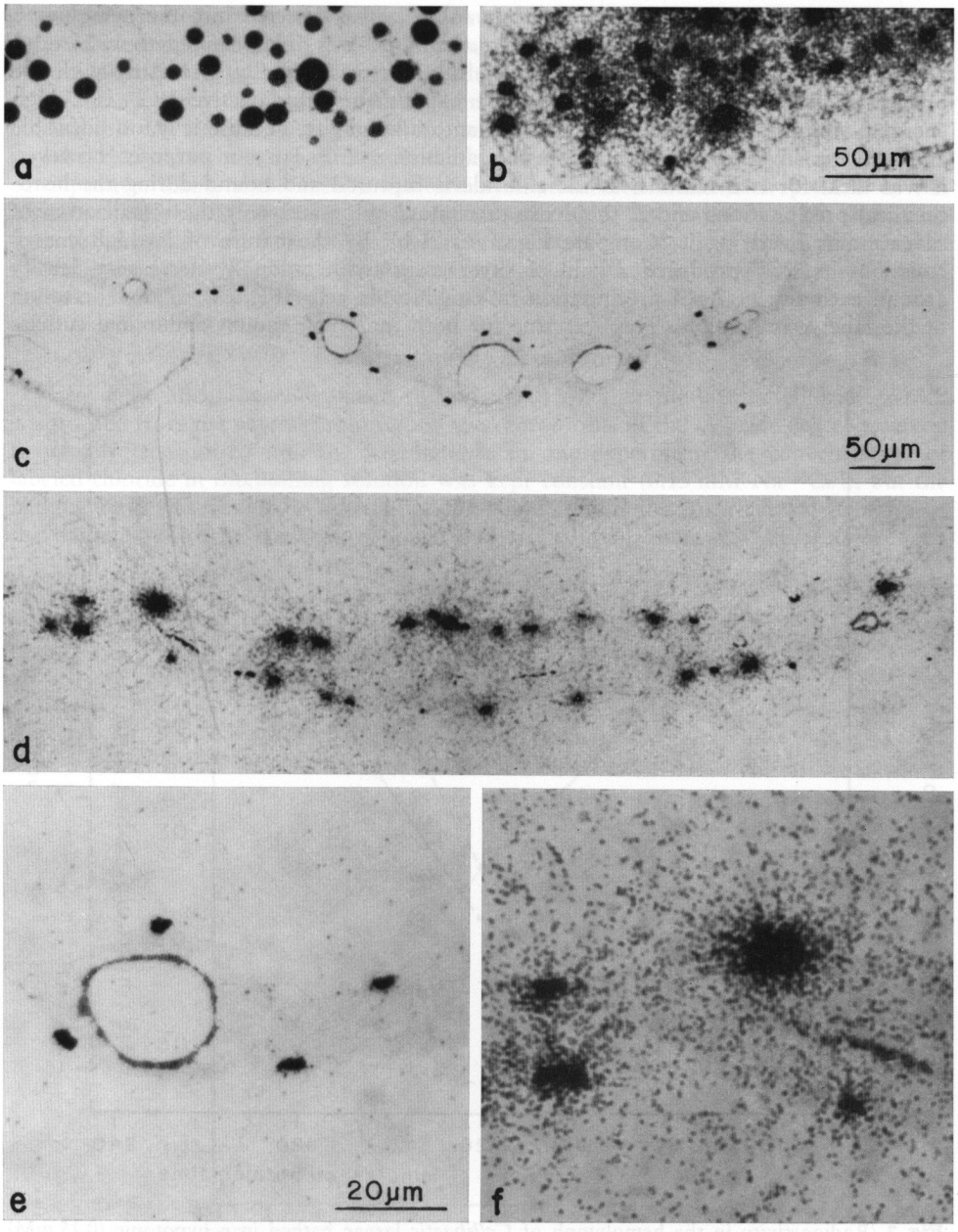
Results and Interpretations

The fine structure and distribution of the chloride cells in *Callibaetis* larvae are very similar to the chloride cell complexes of *Cloeon dipterum* [19] and are described elsewhere [9]. To understand the results of the experiments described in the present paper, it is only necessary to point out that the apex of the central cell of the chloride cell complex is filled with a dense silver chloride precipitation after fixation with the osmium tetroxide-silver lactate solution for histochemical demonstration of chloride (Fig. 1 a). Since the tracheal gills of *Callibaetis* are very flat transparent leaflets, the precipitates are readily visualized with the bright field light microscope (Fig. 1 b). They appear as dark round areas, which reflect approximately the size of the porous plates and indicate the location of a chloride cell complex underneath each of them.

Autoradiography

The histochemical test for chloride indicates that there is a large amount of chloride present in the apex of the central cell of the chloride cell complexes. To study the origin of this chloride, a hypotonic solution of labeled chloride was applied to the

Fig. 2. Autoradiographs of the chloride cells of *Callibaetis* fixed in the histochemical chloride reagent. – **a.** Surface view on the isolated abdominal cuticle of a control specimen bathed in a 15 mM NaCl solution for 1 hour. Exposure time 1 week. – **b.** The same of an experimental animal bathed in 15 mM Na^{36}Cl solution for 1 hour. – 300 ×. – **c.** Cross section through



a tracheal gill of an animal injected with a hypertonic (180 mM) $Na^{36}Cl$ solution and fixed in the histochemical chloride reagent 1 hour after injection. Exposure time 1 month. – **d.** Cross section through a tracheal gill of an animal bathed in a hypotonic (15 mM) $Na^{36}Cl$ solution for 1 hour and fixed in the histochemical chloride reagent. Exposure time 1 month. – 200 ×. – **e.** Higher magnification of c.; **f.** Higher magnification of d. – 700 ×

outside of several larvae and a hypertonic solution was injected into the hemolymph of others. The histochemical precipitation technique was then combined with autoradiography to detect whether the labeled chloride was actually associated with the precipitate in either one or both of these experiments. Radioactive $^{36}Cl^-$ is a high energy β-emitter and not normally used for autoradiography, because it is too diffusible and activates silver grains too far from the radiation source. For our purposes, however, it was ideal for two reasons: 1. the chloride is captured and bound during the histochemical precipitations and 2. the dense precipitate obliterates only the visualization of silver grains overlying it (Compare Fig. 2 a and b). By the nature of its high energy emission the $^{36}Cl^-$ produced a halo of silver grains with gradually decreasing density around each site of AgCl precipitation in the chloride cells (Fig. 2 f) so that detection of the label was possible. This was true for both the whole-mount abdominal cuticles

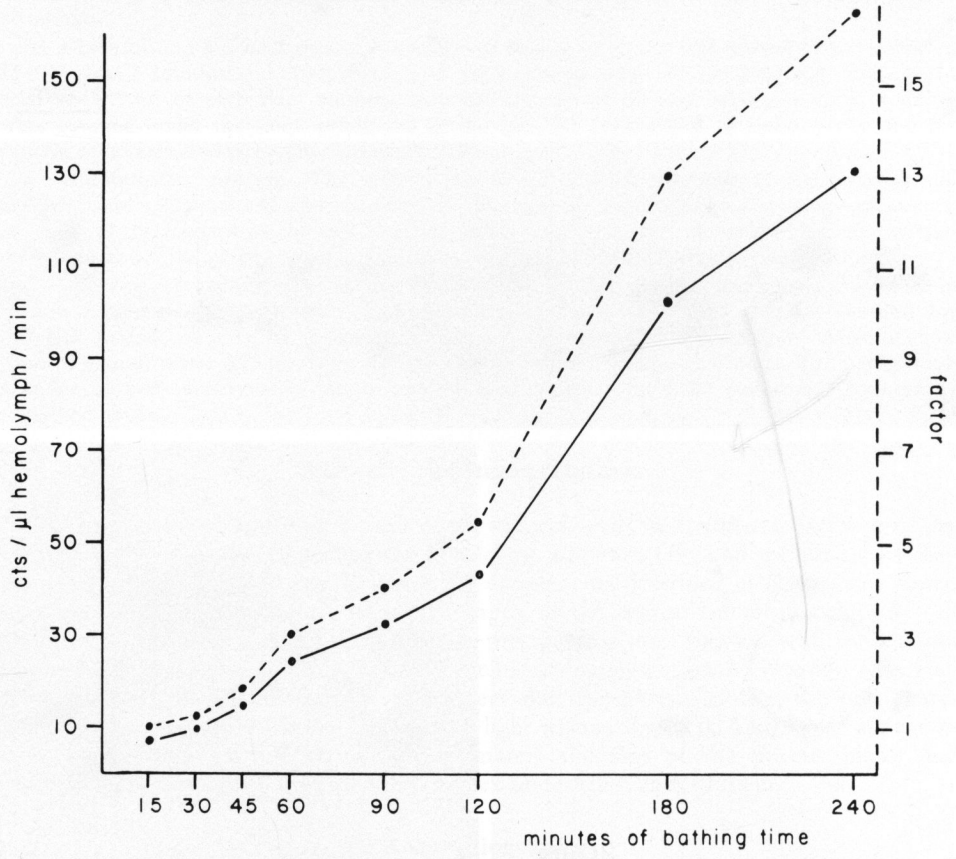


Fig. 3. Radioactivity in the hemolymph of *Callibaetic* larvae bathed in a hypotonic (0.15 mM) $Na^{36}Cl$ solution for different periods of time (full curve). The salt concentration chosen corresponds approximately to the salt concentration of natural water and 10^{-3} of the hemolymph osmolarity. The dotted curve represents the factor

$$\frac{\text{counts/µl hemolymph/minute}}{\text{counts/µl external solution/minute}}$$

and cross sections of the tracheal gills from animals, which had been bathed in hypotonic labeled chloride solution (Fig. 2 b, d and f). By comparison, control preparations of larvae bathed in unlabeled chloride solution contained no label over the chloride cells (Fig. 2 a). In cross section of the gills taken from animals injected with hypertonic labeled chloride solution (Fig. 2 c and e) there was, except for some background grains, no label detectable in association with the histochemical precipitate even after 3 months of exposure. Thus, these results indicate a) that labeled chloride is present in the histochemical chloride precipitates in the chloride cells, b) that chloride is adsorbed from the external media and from hypotonic concentration by the chloride cells, c) that the ephemerid chloride cells, opposite to fish chloride cells, are probably not capable of reversing functional polarity and excreting chloride.

Counting of the Hemolymph

These experiments were designed to investigate whether the labeled chloride, which is adsorbed from the external solution by the central cells of the chloride cell complexes, is actually transported into the hemolymph. In one experiment the concentration of labeled chloride in the bathing solution was kept constant (0.15 mM Na ^{36}Cl/l), but the bathing time was varied between 15 minutes and four hours. In a second experiment the bathing time was constant (1 hour), and the concentration was changed between

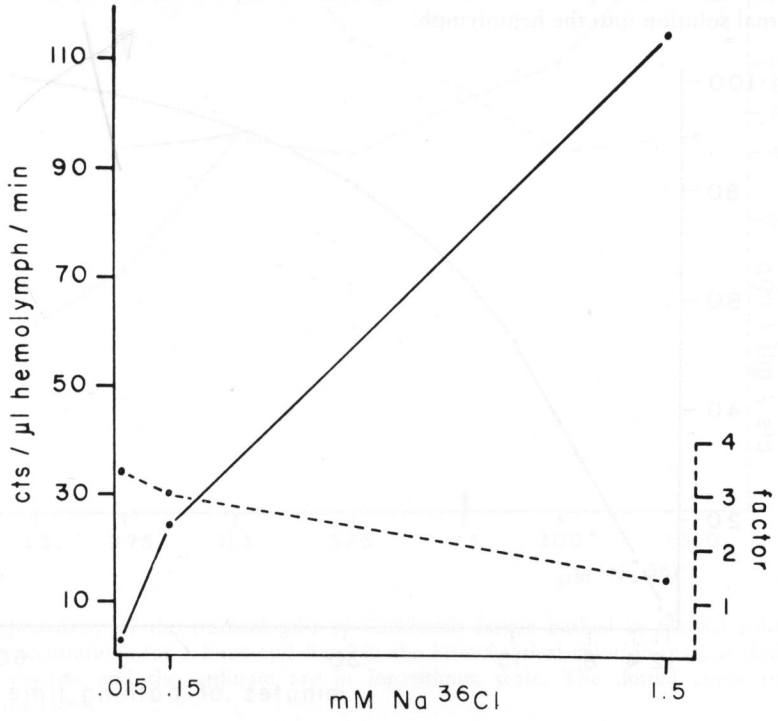


Fig. 4. Radioactivity in the hemolymph of *Callibaetis* larvae bathed in Na^{36}Cl solutions of different concentrations for 1 hour (full curve). The concentrations chosen correspond to 10^{-4}, 10^{-3} and 10^{-2} of the hemolymph osmolarity. The dotted curve represents the same factor as in fig. 3.

0.015 and 1.5 mM Na ^{36}Cl/l. This was done to investigate whether there are any correlations between chloride absorption and bathing time or external concentrations. The results in turn would indicate whether the chloride cells are involved in osmoregulation. Since the total osmolarity of the hemolymph of mayfly larvae may correspond to 148 mM NaCl/l and the chloride concentration may reach 110 meq./l [12], the concentrations of the bathing solutions used in these experiments are extremely hypotonic.

In larvae bathed in 0.15 mM Na ^{36}Cl solution, which is approximately the salt concentration of the natural water used and corresponding to $^{1}/_{1000}$ of the hemolymph osmolarity, an increase of labeled chloride was found within the hemolymph with increased bathing times (Fig. 3). Furthermore, the concentration of labeled chloride within the hemolymph was equal to that of the external solution after only 15 minutes of bathing time (Fig. 3, dotted line). After 30 minutes of bathing time, the concentration of label in the hemolymph exceeds the concentration of label in the bathing solution, and by 4 hours, there is 16.5 times more radioactivity per µl hemolymph than per µl external solution. Assuming that the hemolymph chloride concentration is constant these data indicate that about $^{1}/_{50}$ of the hemolymph chloride is exchanged in 4 hours. Since the concentration of chloride in the labeling solution is only 10^{-3} of the chloride concentration of the hemolymph, the observed net transfer of labeled chloride shows that there is an active absorption of chloride against a steep concentration gradient from the external solution into the hemolymph.

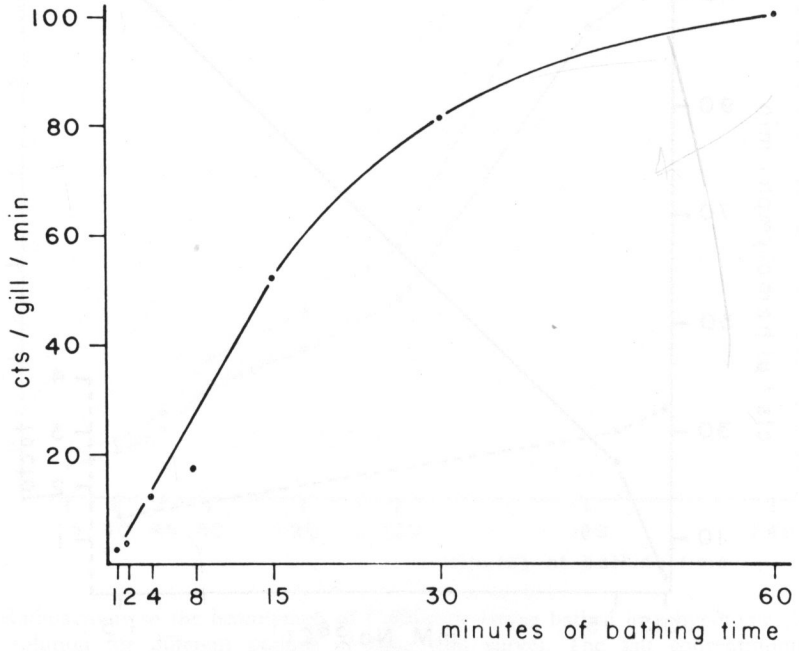


Fig. 5. Radioactivity of the tracheal gills of *Callibaetis* larvae bathed in 15 mM Na^{36}Cl solution for different periods of time and fixed in the histochemical chloride reagent. The NaCl concentration of the bathing solution corresponds to approximately 1/10 of the hemolymph osmolarity. The curve gives the impression of a saturation curve.

In the experiments where larvae were bathed in varying hypotonic salt solutions for 1 hour, there was also an increase of labeled chloride within the hemolymph with increasing salt concentrations of the bathing solutions (Fig. 4). But when compared to the radioactivity of the bathing solutions, there is a decrease in relative chloride uptake with increasing external salt concentration (Fig. 4, dotted line). This means that relatively more chloride is absorbed from more hypotonic than from less hypotonic external salt concentrations. Furthermore, it indicates that the chloride absorption by the chloride cells is involved in osmoregulation. This conclusion of course assumes that the labeled chloride found in the hemolymph is actually derived from the chloride adsorbed by the central cell apex and that it is transported into the hemolymph by the chloride cell complexes. For completeness, two more possibilities must be taken into consideration, 1. passive influx of chloride through external or internal body surfaces and 2. active absorption of chloride somewhere along the intestine after drinking of

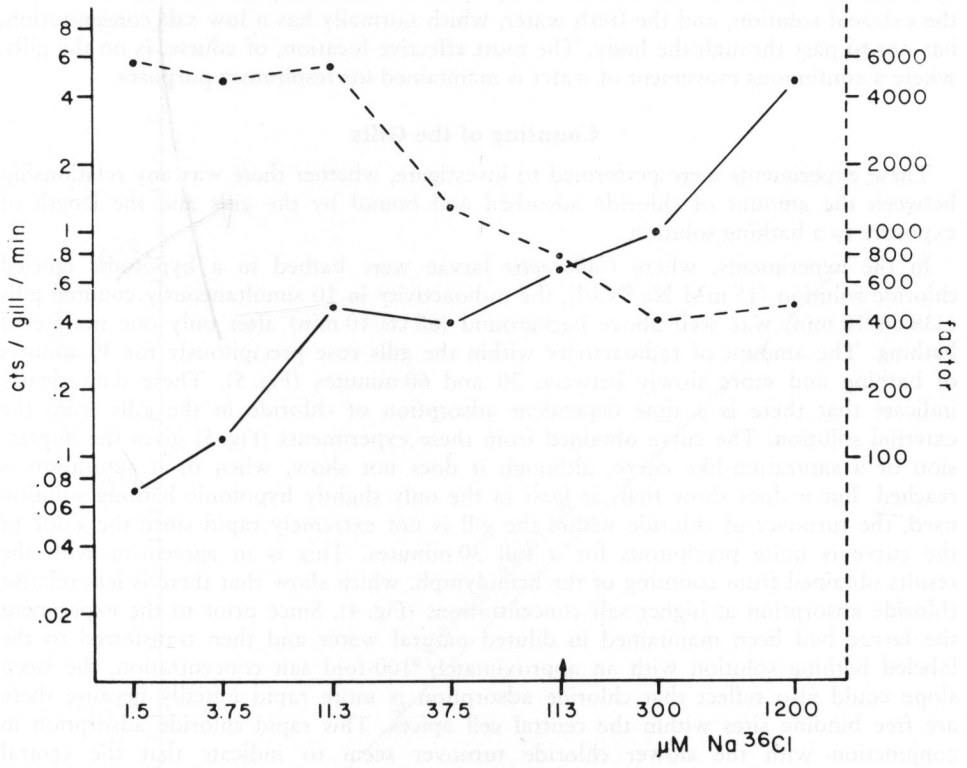


Fig. 6. Radioactivity of the tracheal gills of *Callibaetis* larvae bathed in $Na^{36}Cl$ solutions of different concentrations for 1 hour and fixed in the histochemical chloride reagent (full curve). Both the abscissa and the ordinate are in logarithmic scale. The dotted curve represents the following factor

$$\frac{\text{counts/gill/minute}}{\text{counts/}1.5 \times 10^{-4}\ \mu\text{l of bathing solution/minute}}$$

1.5×10^{-4} μl is the approximate volume of silver chloride precipitate per gill. The *arrow* indicates the natural water salt concentration.

water. Net influx of chloride by diffusion can be excluded under the extremely hypotonic conditions used in these experiments. The possibility of active absorption by the intestine can also be excluded by a simple calculation: The larvae, for example, which were bathed in 0.015 mM NaCl solution, would have to drink 3.5 times their total hemolymph volume within 1 hour and quantitatively absorb all the chloride ions therein to account for the net chloride uptake found. This appears to be quite unreasonable, because this much water would greatly dilute the intestinal contents and reduce the nutritional effectiveness. On the other hand, both the adsorption of chloride by the central cell apex (Fig. 2 b and d) and the fine structural feature of the chloride cells [9] strongly suggest that the chloride cells are responsible for absorption of chloride from the external solution and its transport into the hemolymph.

At the same time, these simple calculations also seem to explain why in fresh water animals (e. g., mayfly larvae, mosquito larvae, fish) chloride cells and salt absorbing epithelia are located at the external body surface. There they are directly exposed to the external solution, and the fresh water, which normally has a low salt concentration, has not to pass through the body. The most effective location, of course, is on the gills, where a continuous movement of water is maintained for respiratory purposes.

Counting of the Gills

These experiments were performed to investigate, whether there was any relationship between the amount of chloride adsorbed and bound by the gills and the length of exposure to a bathing solution.

In the experiments, where *Callibaetis* larvae were bathed in a hypotonic labeled chloride solution (15 mM Na ^{36}Cl/l), the radioactivity in 10 simultaneously counted gills (338 cts/10 min) was well above background (60 cts/10 min) after only one minute of bathing. The amount of radioactivity within the gills rose precipitously for 30 minutes of bathing and more slowly between 30 and 60 minutes (Fig. 5). These data clearly indicate that there is a time dependent adsorption of chloride in the gills from the external solution. The curve obtained from these experiments (Fig. 5) gives the impression of a saturation-like curve, although it does not show, when or if saturation is reached. But it does show that, at least in the only slightly hypotonic bathing solution used, the turnover of chloride within the gill is not extremely rapid since the slope of the curve is quite precipitous for a full 30 minutes. This is in agreement with the results obtained from counting of the hemolymph, which show that there is less relative chloride absorption at higher salt concentrations (Fig. 4). Since prior to the experiment the larvae had been maintained in diluted natural water and then transferred to the labeled bathing solution with an approximately 100-fold salt concentration, the steep slope could also reflect that chloride adsorption is more rapid initially because there are free binding sites within the central cell apices. This rapid chloride adsorption in conjunction with the slower chloride turnover seem to indicate that the central cell apices constitute a pool of chloride storage and adsorb more chloride than is immediately needed and used for osmoregulation.

Finally, experiments were run to determine the absorptive capacity of the chloride cells, when the larvae were exposed to more dilute salt concentrations. The labeled

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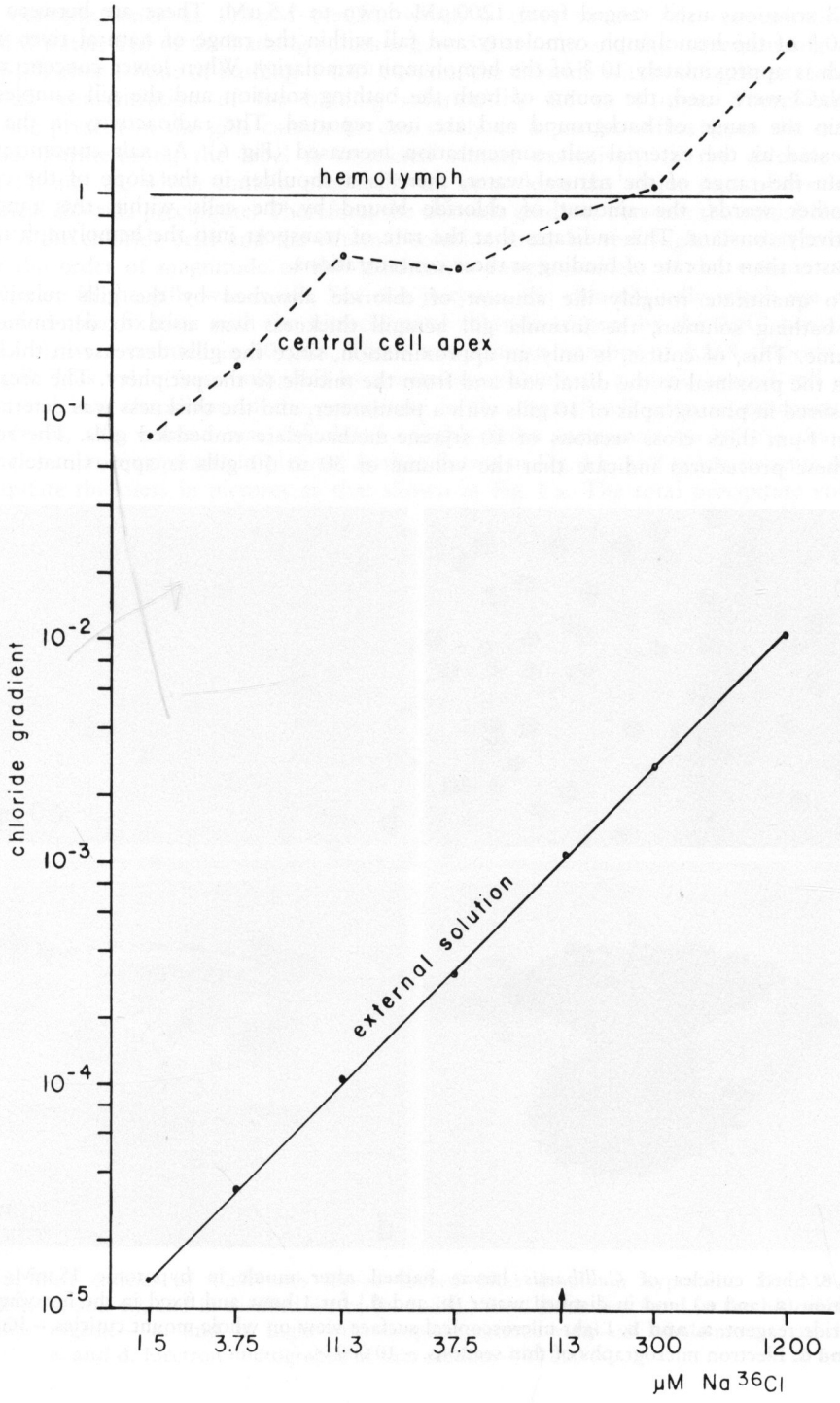
Fig. 7. Chloride gradients between external bathing solution and hemolymph, and between chloride cell apices and hemolymph as calculated from the results in figure 6. The arrow indicates the natural water salt concentration. – Double logarithmic scale.


hemolymph
central cell apex
external solution
chloride gradient
1
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
1.5
3.75
11.3
37.5
113
300
1200
μM $Na^{36}Cl$

NaCl solutions used ranged from 1200 µM down to 1.5 µM. These are between 10^{-2} to 10^{-5} of the hemolymph osmolarity and fall within the range of natural river water which is approximately 10^{-3} of the hemolymph osmolarity. When lower concentrations of NaCl were used, the counts of both the bathing solution and the gill samples fell within the range of background and are not reported. The radioactivity in the gills increased as the external salt concentration increased (Fig. 6). At salt concentrations within the range of the natural water, there is a shoulder in the slope of the curve. In other words, the amount of chloride bound by the cells within this range is relatively constant. This indicates that the rate of transport into the hemolymph might be faster than the rate of binding at these concentrations.

To quantitate roughly the amount of chloride absorbed by the gills relative to the bathing solution, the formula gill aeragill thickness was used to determine gill volume. This, of course, is only an approximation, since the gills decrease in thickness from the proximal to the distal end and from the middle to the periphery. The area was measured in photographs of 10 gills with a planimeter, and the thickness was determined from 1 µm thick cross sections of 10 styrene-methacrylate embedded gills. The results of these procedures indicate that the volume of 30 to 50 gills is approximately 1 µl.

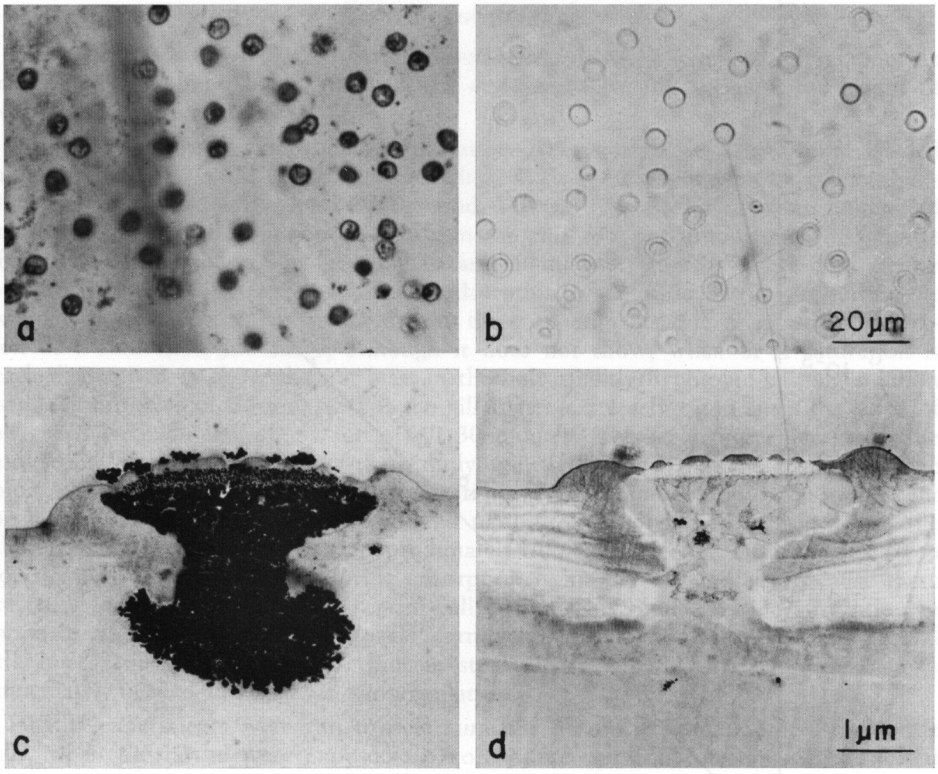


Fig. 8. Shed cuticles of *Callibaetis* larvae bathed after moult in hypotonic 15 mM NaCl solution (**a.** and **c.**), and in distilled water (**b.** and **d.**) for 1 hour and fixed in the histochemical chloride reagent. **a. and b.** Light microscopical surface view on whole-mount cuticles. – 500 ×. – **c. and d.** Electron micrographs of thin sections. – 10 000 ×.

For example, since at 113 μM NaCl/l, which is close to the salt concentration of natural water, 1 μl of the bathing solution gave 5.8 cts/min and the corresponding value for 1 gill after 1 hour of bathing is 0.69 cts/min, the chloride within the gills exceeds the chloride concentration of the bathing solution by 3.5 to 6 times. Therefore, the chloride present in the gills cannot be due solely to a passive influx from the outside media. Furthermore, the label is restricted almost exclusively to the chloride cells (Fig. 2 b and d), which contain practically all or at least the major part of the histochemical chloride precipitate. Therefore, the chloride gradient between the actual binding sites in the chloride cells and the external solution must be very high. To determine at least the order of magnitude of this gradient, a rough estimate of the volume of the precipitate in the gills was made. For this purpose, the chloride cells which are easily identified after fixation with the histochemical chloride reagent by the AgCl precipitate (Fig. 1 b), were counted in 10 gills. There was a mean number of 1,568 chloride cells per gill. This value was multiplied by precipitate volume in a single central cell apex. Assuming that the shape of the precipitate in the central cell apex is approximately cylindrical, this volume was calculated roughly from measurements of the diameter of the circular precipitates in pictures as that shown in Fig. 1 b, and measurements of the precipitate thickness in pictures as that shown in Fig. 1 a. The total precipitate volume

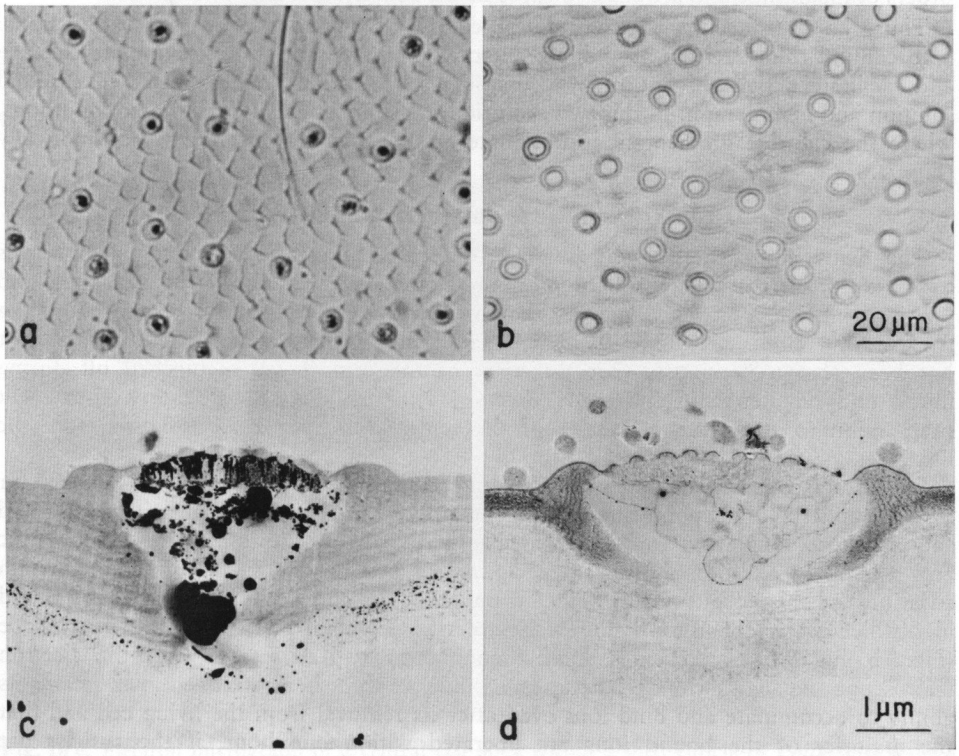


Fig. 9. Shed cuticles of *Callibaetis* larvae bathed after moult in hypotonic 15 mM NaCl solution (**a.** and **c.**), and in distilled water (**b.** and **d.**) for 1 hour and fixed in the histochemical sodium reagent. – **a. and b.** Light microscopical surface view on whole-mount cuticles. – 500 ×. – **c. and d.** Electron micrographs of thin section. – 10 000 ×.

per gill was found to be about $1.5 \cdot 10^{-4}$ µl and was used as a basis for the comparison of the counts per gill and the counts per bathing solution of corresponding experimental groups. The results are demonstrated in the dotted factor-curve in Fig. 6. This factor expresses how much more radioactivity was found in the silver chloride precipitate per gill than in a corresponding volume of bathing solution. Based on these calculations the amount of labeled chloride within the central cell apices of the chloride cell complexes was found to be 500 to 6000 times greater than within the external solutions depending on the salt concentrations used. The factor is highest at the lowest external concentration, and decreases as the external concentration is increased. This means that the *relative* adsorption decreases with increasing external salt concentrations, and indicates that the turnover of chloride in the central cell apex is slowed down at higher external salt concentrations. A corresponding tendency was found for the relative absorption of chloride into the hemolymph (Fig. 4).

In Figure 7 the approximate amount of chloride present in the central cell apices and the chloride concentrations of the bathing solutions are plotted as chloride gradients of the hemolymph chloride concentration. Under the experimental conditions used the chloride gradients of the bathing solutions range between 10^{-5} and 10^{-2}, whereas the corresponding values for the central cell apex are lifted by approximately three orders of magnitude and are close to those of the hemolymph (Fig. 7).

Thus, the central cell of the chloride cell complex seems to have two major functions: 1. to capture chloride from very dilute external solutions, and 2. to accumulate chloride and facilitate active absorption by decreasing the chloride gradient between external solution and hemolymph. In this way, chloride transport under natural water conditions, where the chloride gradient is approximately 10^{-3}, might occur nearly isoosmotically (Fig. 7).

Properties of the Isolated Central Cell Apex

The dense silver chloride precipitate observed within the central cell apex seems to indicate that the apex probably contains some material capable of attracting and binding chloride ions. Furthermore, the time dependent adsorption curve resembling a saturation curve suggests that this material has ion exchange properties. Since it has been shown previously that the apex of the central cell is pinched off during moult and remains with the shed cuticles [19], fresh exuviae provide a convenient preparation to study the properties of the central cell apex after isolation from the living cell.

In cuticles collected immediately after moult, bathed in 15 mM NaCl solution for 1 hour, and fixed with the histochemical methods for demonstration of chloride and sodium, both ions were found to be absorbed by the central cell apex (Fig. 8 a, c and 9 a, c). In addition the dense precipitate of both Na and Cl were found within striations of the porous plates (Fig. 8 c and 9 c). In cuticles, which had been rinsed in distilled water for 1 hour, no precipitate was detectable with the light microscope (Fig. 8 b and 9 b), however, a small amount was still detectable with the electron microscope (Fig. 8 d and 9 d). These results indicate that the central cell apex retains its ability to accumulate and bind ions even after its removal from the living cell and that the majority of the bound ions are liberated within one hour, if the cuticles are bathed in distilled water. In cuticles, which had been rinsed in distilled water first and then bathed in NaCl solution, however, the chloride precipitates were visible with the light microscope. Therefore, the loss of bound ions into the distilled water is not due to a loss of binding material, but caused by some sort of an ion exchange.

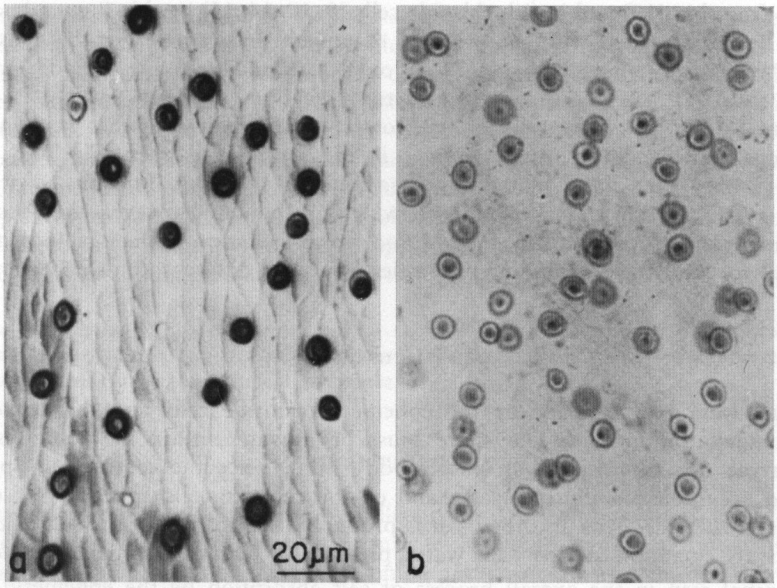


Fig. 10. Surface views of shed cuticles of *Callibaetis* larvae fixed immediately after moult in 3 % buffered glutaraldehyde and stained with (**a**) Hale's colloidal iron (abdominal cuticle with brown pigment) and (**b**) PAS (gill cuticle). – 500 ×.

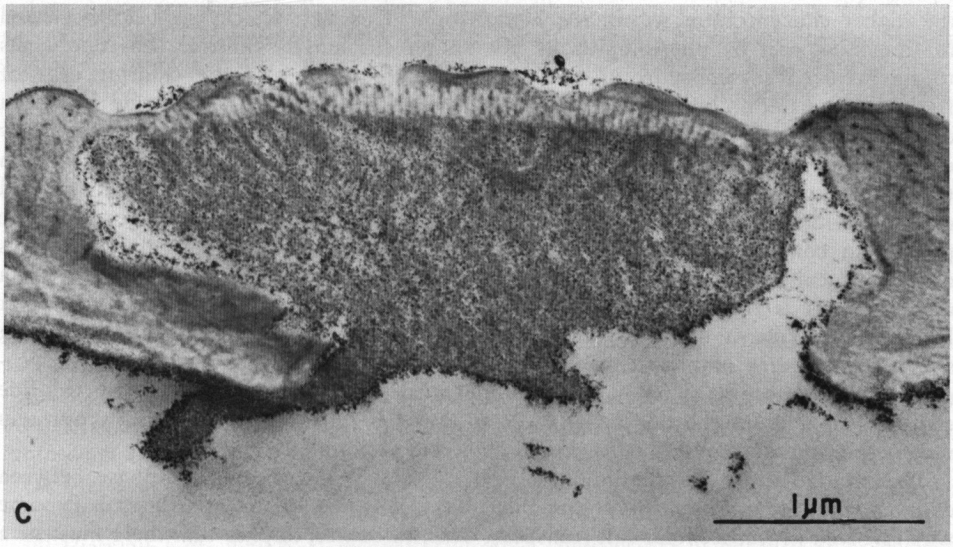


Fig. 10 c. Electron micrograph of a central cell apex within a shed cuticle of *Callibaetis* stained in block with colloidal iron at *p*H 2.5. – 28 000 ×.

In the frog skin [18] and in fish chloride cells [3, 10, 14, 15] ion binding and accumulation has been correlated with the presence of mucoid substances. For this reason, shed cuticles were also stained with Hale's colloidal iron and with the periodic acid Schiff reagent (PAS). The colloidal iron gave a strong reaction (Fig. 10 a) and the PAS stain a weak one (Fig. 10 b) at the sites of the porous plates indicating that acid mucopolysaccharides and glycoproteins are present in the central cell apex (Fig. 10 c). This in no way proves that these materials are actually responsible for ion adsorption or whether it is primarily cations or anions, which are bound. However, the shed cuticles presumably provide a suitable means for biochemical isolation of the ion binding material as well as for studying the binding properties by use of different kinds and combination of ions.

Discussion

The absorption of chloride by the ephemerid chloride cells and their involvement in osmoregulation indicate that there must be a water influx in these fresh water insect larvae due to their hyperosmotic body fluid. Since the central cell apices also bind and accumulate sodium (Fig. 9 a and c), the chloride cells appear to absorb both sodium and chloride. This absorbed salt is probably used to replace the salt lost through the necessary excretion of the excess water, presumably by the Malpighian tubules.

The initial step in the overall process of salt absorption by the ephemerid chloride cells is the catching, binding, and accumulation of ions from dilute concentrations by the central cell apex. This step, which is independent of the direct action of the living cell and due to chemical properties of some unidentified material, creates a pool of bound ions between the extremely low salt concentration of the external solution and the higher concentration within the hemolymph. This step which has been neglected for the most part by physiologists, is not unique for mayfly chloride cells but seems to be involved in transport of salt across the frog skin and the chloride cells of fresh water fish. In both cases, an accumulation of sodium and chloride has been demonstrated histochemically within external mucoid layers [3, 10, 18]). The only difference in the ephemerid chloride cells is that the main accumulation site is on the inside surface of the cell apex. However, the demonstration of sodium and chloride within the porous plates themselves (Fig. 8 c and 9 c) indicates that the pores also contain some ion binding and accumulating material (see also [9]). This material is exposed directly to the external medium and not separated by the plasma membrane as is the binding material within the central cell apex. Although direct biochemical evidence is still lacking, the correspondence of the main ion binding sites with the sites of histochemical staining for acid mucopolysaccharides and glycoproteins, which are at different faces of the apical plasma membrane in fish chloride cells and ephemerid chloride cells, suggests that the mucopolysaccharide glycoprotein moiety seems to be responsible for ion binding and accumulation [2, 13].

The accumulation of large amounts of ions (Fig. 7) just at the interphase between external solution and hemolymph apparently indicates that this compartment is in some way involved in transcellular salt transport. This, of course, implies that there must be a release mechanism to liberate the bound ions and make them available for transport [18], even if there is an atmosphere of free ions in addition to the bound ones [7]. Since the bound ions can be liberated simply by rinsing in distilled water (Fig. 8 and 9) the primary mechanism might be one of ion exchange requiring OH^- if anions are bound or H^+ if cations are bound. These could be provided by the mitochondria of

the chloride cells. The release of bound ions would increase the ion concentration within the central cell, which in turn would facilitate their further transport into the hemolymph. The numerous mitochondria of the chloride cells seem to exclude the possibility that the transport is merely diffusion but the ions could ar least diffuse down the central cell and perhaps also into the adjacent cells. However, an increased ion concentration would reduce the energy requirements for active salt transport from the cells into the hemolymph. Since the interdigitation of the adjacent cells of the chloride cell complexes provides intercellular channels that open towards the base [9], the end phase of the overall transport process in ephemerid chloride cell complexes might be explained by the standing gradient theory [4 to 6].

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Zusammenfassung

In Versuchen mit radioaktiven Chloridlösungen an *Callibaetis*-Larven *(Ephemeroptera, Baetidae),* in denen die histochemische Methode zum Chloridnachweis mit der Autoradiographie kombiniert wurde, konnte nachgewiesen werden, daß die Chloridzellen dieser Tiere Chlorid aus hypotonischem Milieu absorbieren. Ferner wurde durch Radioaktivitätsmessungen der Hämolymphe und der Tracheenkiemen nach histochemischer Chloridfällung gezeigt, daß diese Absorption zeit- und konzentrationsabhängig ist und daß die Chloridzellen an der Osmoregulation beteiligt sind.

Der Apex der Zentralzelle in den Chloridzellkomplexen enthält saure Mucopolysaccharide und Glycoproteine und vermag Natrium und Chlorid aus hypotonischen Lösungen zu binden und zu akkumulieren. Diese Fähigkeit ist unabhängig von der lebenden Zelle. Die Bedeutung der Elektrolytakkumulation im Apex der Zentralzelle, also zwischen äußerem Milieu und Hämolymphe, wird im Hinblick auf die Salzaufnahme aus Süßwasser und den transzellulären Salztransport erörtert.

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