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OSMOREGULATORY ROLE AND TRANSPORT ATPASES OF THE RECTUM OF DRAGONFLY LARVAE*

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The use of radioactive salt solutions in ligation experiments has revealed that the larvae of both Zygoptera and Anisoptera absorb Na^+ and Cl^- ions through the rectum from highly hypo-osmotic concentrations. In Zygoptera, there are 3, in Anisoptera up to about 500 epithelial pads in the rectum which show fine structural features of transporting epithelia. Rectal ventilation provides contact of these epithelia to the external medium from where the ions are absorbed. The results of histochemical chloride precipitation in conjunction with X-ray analysis, autoradiography, and liquid scintillation counting suggest that these pads, termed rectal chloride epithelia, are the sites specialized for ion uptake. Activities of Na/K- and HCO_3-ATPase are biochemically demonstrated in homogenates of the rectum of *Aeshna cyanea.* The highest activities of both enzymes are found in the membrane fraction. Long-term acclimatization of larvae to different environmental salinities results in different sizes of the chloride epithelia and concomitant changes of ATPase activity. The significant increase in epithelial size and activities of the transport ATPases in highly hypo-osmotic, nearly salt-free medium is consistent with the enhanced osmoregulatory activity required under these conditions.

OSMOREGULATORY SITUATION

Like many other freshwater insects so far studied (STOBBART & SHAW, 1974) dragonflies are hyperosmotic regulators (SUTCLIFFE, 1962), i.e. the osmolarity of the hemolymph is maintained well above the osmotic concentration of the environmental medium. In *Aeshna cyanea* larvae for example, the he-

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molymph is highly hyperosmotic to freshwater and kept nearly constant over a wide range of environmental salinities (KOMNICK, 1977b). When the external osmolarity is experimentally increased to values exceeding the nominal level of the hemolymph osmolarity the latter is raised nearly parallel until the animal dies at more than 600 mosm (Fig. 1). These data indicate that the larvae have a good capability of hyperosmotic regulation at low concentration, but they have little, if any, osmoregulatory ability at originally hyperosmotic concentrations where they almost behave as osmoconformers.

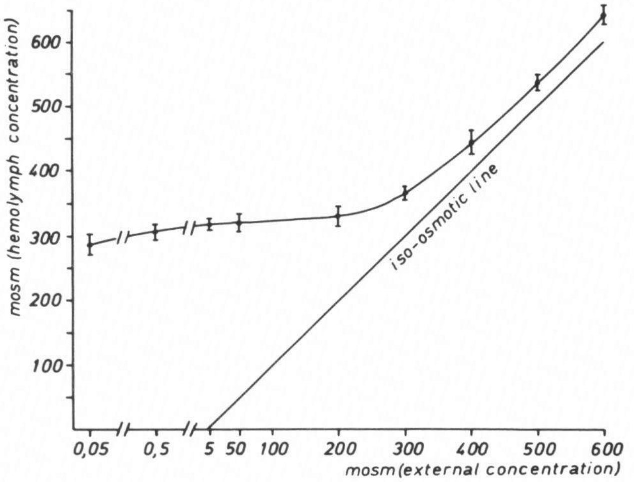

Fig. 1. Osmotic concentration of the hemolymph of *Aeshna cyanea* after 10 days in different concentrations of natural sea salt. Each value represents mean ± S.D. of 10 unfed larvae of 35 mm body length.

MOENS (1975) has shown that the concentrations of sodium and chloride which contribute to about 70% of the hemolymph osmolarity, follow the same curve pattern as the hemolymph osmolarity. This means that hyperosmotic regulation is mostly due to the regulation of these ions. In freshwater environments a steep gradient exists between the internal and external solutions which favors passive ion loss and osmotic water influx. The excess water entering osmotically must be eliminated by the production of hypo-osmotic urine, which causes further loss of ions. Consequently, active absorption of ions from the low-concentrated freshwater environment is required for the positive ionic balance of the hemolymph. This raises the question which organ or cells perform osmoregulatory ion uptake.

EVIDENCE OF RECTAL ION ABSORPTION

KOCH (1934) was the first to show that the epithelial pads in the rectal wall of both anisopterous and zygopterous larvae reacted with silver salts added to the medium. He assumed that the silver ions were precipitated by chloride ions at these sites and that the epithelial pads were involved in osmoregulatory

Table I

Radioactivity measured in 2.5 µl hemolymph samples taken from 2 experimental groups (N = 10) of *Coenagrion puella* larvae after 3 hours in hypo-osmotic radioactive salt solutions.

External solution	Isotope	Anus	cpm/µl hemolymph
1 mM NaCl	0.1 µci $^{22}Na^+$/ml	open	123.0 ± 33.2
		ligated	1.3 ± 1.1
1 mM NaCl	0.1 µci $^{36}Cl^-$/ml	open	362.0 ± 124.0
		ligated	3.5 ± 0.6

ion uptake. However in the past, this possible function of the odonate rectum was widely neglected.

In order to provide direct evidence for the osmoregulatory role of the rectum in both zygopterans and anisopterans, we have studied *Coenagrion puella* and *Aeshna cyanea* larvae with the use of radioisotopes (SCHMITZ & KOMNICK, 1976). The larvae were placed in hypo-osmotic 1 mM NaCl solutions

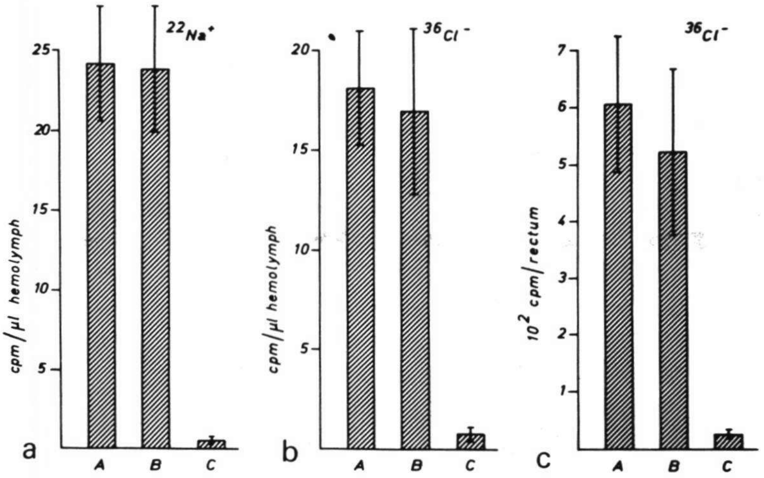

Fig. 2. *Aeshna cyanea*: (a-b) Radioactivity measured in 20 µl hemolymph samples withdrawn from 3 groups of larvae (N = 5) after 6 hours in hypo-osmotic (a) 1 mM ^{22}NaCl (0.01 µci/ml) and (b) 1 mM Na ^{36}Cl (0.01 µci/ml); – (c) Radioactivity of the re-dissolved silver chloride precipitates of the rectal chloride epithelia in larvae of equal body length after 6 hours in 1 mM Na ^{36}Cl (0.01 µci/ml). N = 5. – (A: untreated larvae; B: larvae with sealed mouth; C: larvae with sealed anus).

containing either radioactive sodium or chloride for a selected period of time. Thereafter hemolymph samples were taken with calibrated micropipettes and measured in the liquid scintillation counter. Prior to the exposure to the labelled solutions the larvae of the two species were divided into the following experimental groups: *C. puella* larvae (1) untreated (control), and (2) with ligatures of the anal opening; *A. cyanea* larvae (1) untreated (control), (2) with paraffin-sealed mouth, and (3) with paraffin-sealed anus. The results presented in Table I and Figure 2a, b clearly indicate that in both species the two kinds of ions important for osmoregulation enter the body via the anal opening and are taken up into the hemolymph through the rectal wall against their steep concentration gradients.

TRANSPORTING EPITHELIA OF THE RECTUM

The rectum of zygopterous and anisopterous larvae is quite different in structure, the former being a rather simple tube (Fig. 3a), the latter constituting a highly complicated branchial chamber (Fig. 4a, c) (cf. also IMMS, 1973). In both cases the rectum is ventilated through the anal opening, and the rectal ventilation was regarded as indicative of a respiratory function. This function is well established now in anisopterans, but in zygopterans it is not. In contrast, PENNAK & McCOLL (1944) observed that, unlike in anisopterans (BABAK & FOUSTKA, 1907), the frequency of rectal ventilation in zygopterans was not related to the oxygen content of the medium. Instead, it was found to be related to the salt concentration of the external medium (Table II) (WICHARD & KOMNICK, 1974c). In conjunction with the results obtained from the experiments with radioactive salt solution it is evident that rectal ventilation serves partly (in anisopterans) or exclusively (in zygopterans) the osmoregulatory function of the rectum in that the ion absorptive structures are thereby exposed to the external medium.

The next question is whether the epithelial pads, which we have termed rectal chloride epithelia, actually represent these ion absorptive sites as formerly assumed by KOCH (1934).

Zygopterous larvae possess three rectal chloride epithelia (Fig. 3a). The cells are coined by numerous folds of the apical and basal cell membranes and by numerous mitochondria (Fig. 3b). This type of cellular fine structure is characteristic of epithelia involved in active ion transport. Anisopterous larvae possess one small and one large chloride epithelium on each basal side of every gill leaflet in addition to six pre-anal pads which are not associated with gills (Figs. 4a-d, 5a). The total number of chloride epithelia in *A. cyanea* increases with the body size and amounts to about 500 in larvae of more than 25 mm in body length (KOMNICK, 1977a). The apical portion of the leaflet is clearly differentiated as tracheal gill (Fig. 5 b), whereas the basal epithelial pads exhibit the fine structural features of ion transporting cells (Fig. 5c) (WICHARD & KOMNICK, 1974a, 1974b) and closely resemble the chloride epithelia of zygopterans.

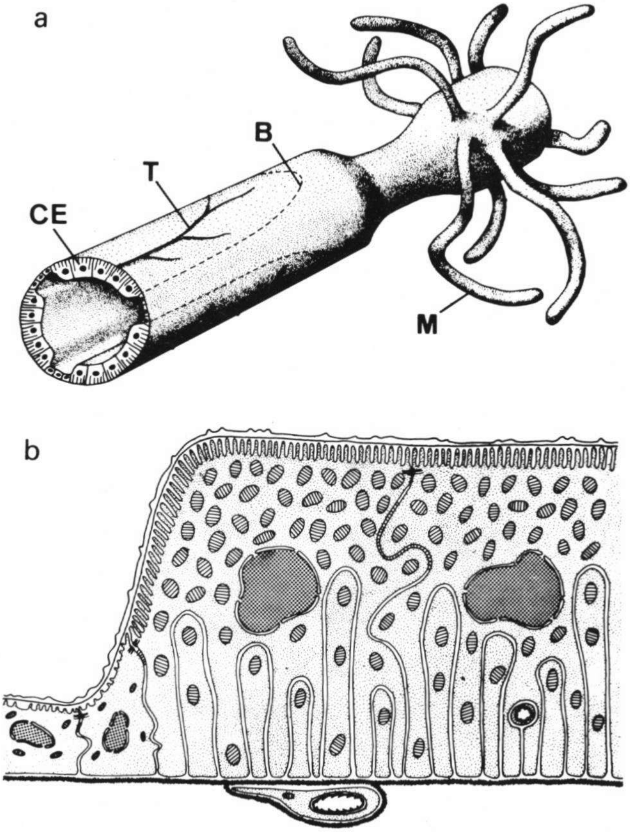

Fig. 3. *Coenagrion puella*: (a) Diagram of the larval hindgut showing the 3 chloride epithelia (CE) in section. (B: border of chloride epithelia; T: trachea; M: Malpighian tubles); – (b) Diagram of the fine structure of the chloride epithelia.

Table II

Frequency of rectal ventilation of *Coenagrion puella* larvae at different external salinities (N = 10; body length 8.5 to 12 mm exclusive the caudal lamellae)

External solution	Numerical range of ventilation cycles observed in 2 hours	Mean number
10^{-5} M NaCl	837 - 2524	1756
10^{-1} M NaCl	0 - 18	7

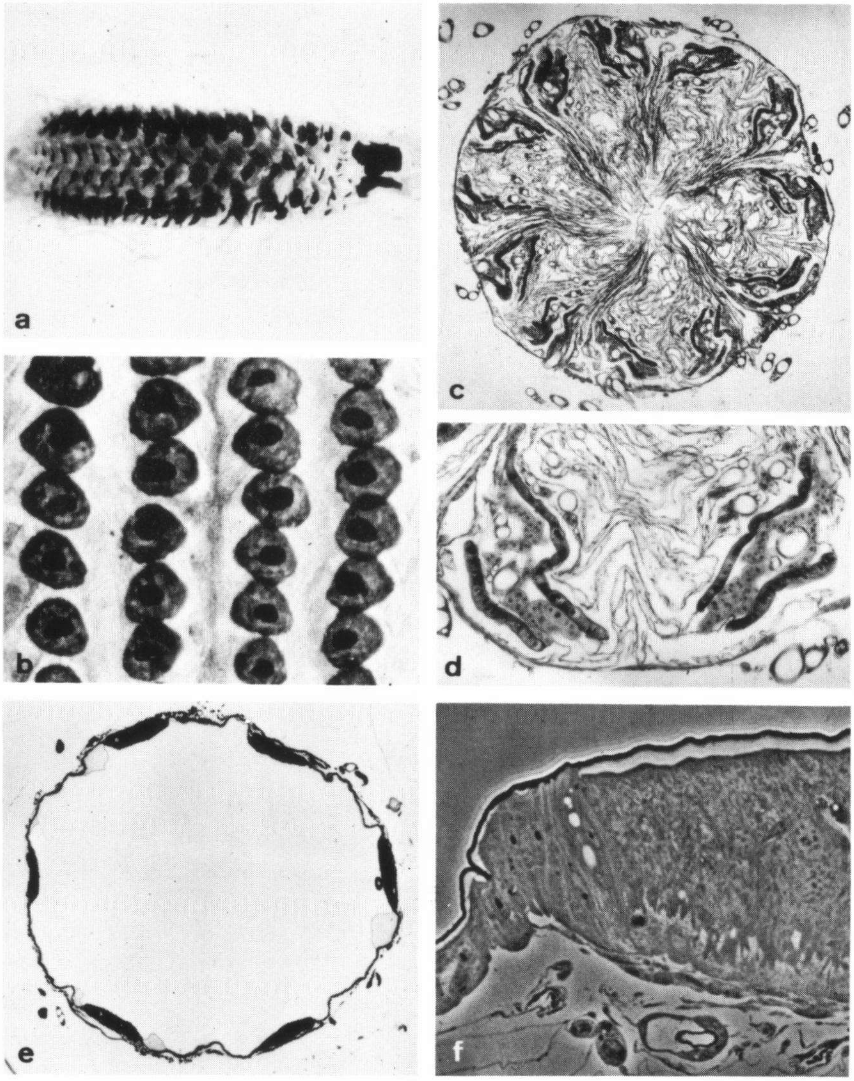

Fig. 4. *Aeshna cyanea*: (a) Whole-mount preparation of the larval rectum showing rows of chloride epithelia and pre-anal pads (right side) as black patches after fixation in the glutaraldehyde-silver nitrate-nitric acid mixture and photochemical reduction of the precipitates. 14 x. – (b) Rectum after the same fixation as above. The isolated rectum was cut lengthwise and mounted on the slide as a flat preparation. The picture shows part of 2 double rows of gill-associated chloride epithelia. The large and small chloride epithelia of each gill leaflet appear in superposition. 27 x; – Paraffin cross section of the larval rectum. The chloride epithelia are darkly stained with PAS due to their content of glycogen (GREVEN

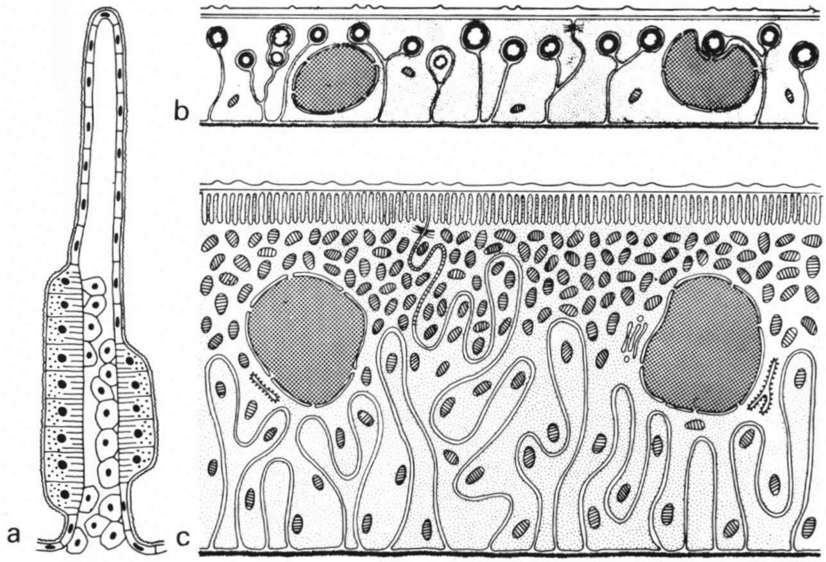

Fig. 5. *Aeshna cyanea*: (a) Schematic presentation of a median section of a gill leaflet, showing the 2 contralateral chloride epithelia in the basal part and the thin respiratory epithelium in the apical part; – (b) Diagram of the fine structure of the branchial epithelium, showing numerous tracheoles closely underneath the cuticle; – (c) Diagram of the fine structure of the rectal chloride epithelia.

After emergence, i.e. when the animals have changed from the aquatic to the terrestrial habitat, all gill associated chloride epithelia have disappeared together with the branchial chamber. In the winged adults only 6 elongated rectal pads are present (Fig. 4e) which according to FAUSSEK (1887) are homologous to the 6 pre-anal pads of the larval rectum. As judged from their structure at the light microscopic level (Fig. 4f), they probably serve the reabsorption of water and/or ions from the excrement and urine as in other terrestrial insects (WALL & OSCHMAN, 1975).

& RUDOLPH, 1973). The 6 double rows each consisting of two chloride epithelia are apparent. The major part of the rectal lumen is occupied by the gill leaflets. 23 x; – (d) Higher magnification showing the contralateral large and small chloride epithelia of 2 gill leaflets. 63 x; – (e) One μm thick cross section of the rectum of an adult fixed 2 days after emergence, showing the 6 rectal pads. Toluidin blue, 30 x; – (f) One μm thick section of the imaginal rectal pad and the adjacent thin part of the rectal wall. Phase contrast, 550 x.

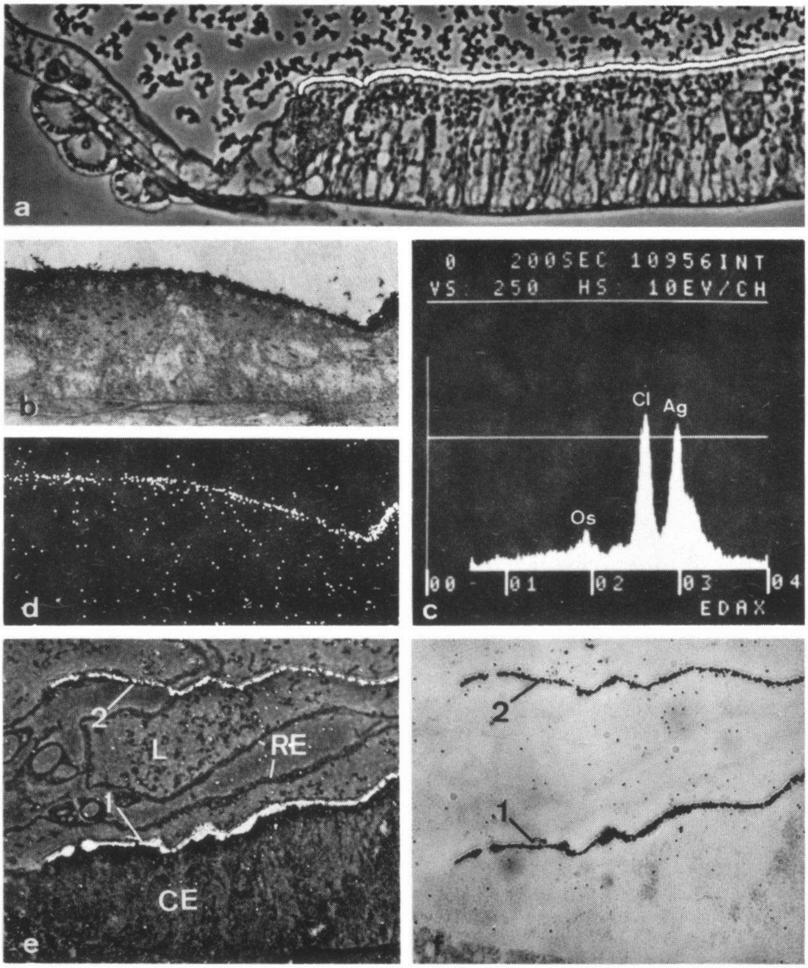

Fig. 6. Chloride histochemistry and autoradiography: (a) *Coenagrion puella*, 1 μm thick section of the hindgut fixed with 5% glutaraldehyde containing 0.1 M silver nitrate and 1 N nitric acid, showing the bright line of silver chloride precipitates at the luminal face of one chloride epithelium. Phase contrast. 750 x. – (b-f) *Aeshna cyanea*, the larval rectum was fixed for histochemical chloride precipitation in 2% osmium tetroxide containing 1% silver lactate: (b) Scanning transmission electron micrograph of a 0,5 μm thick section showing the histochemical precipitates at the luminal face of the chloride epithelium. 1000 x; – (c) X-ray diagram of a spot analysis of the precipitates, showing the presence of osmium, chlorine, and silver; – (d) X-ray distribution image of chlorine K_α of the same area depicted in b; – (e-f) Autoradiograph of a 1 μm thick section of the rectum. Prior to fixation in the osmium/silver solution the larva was kept in 1 mM Na ^{36}Cl (0.1 μci/ml) for 1 day. The artificial shift of the photoemulsion after exposure permits the differentiation between the line of histochemical silver chloride precipitates (1) at the luminal face of the

CHLORIDE HISTOCHEMISTRY OF THE RECTUM

Fixation of the larval rectum with silver salt-containing fixatives results in dense precipitates which specifically line the luminal surface of the chloride epithelia of both *C. puella* and *A. cyanea* (Fig. 6a-d). These precipitates appear white immediately after their formation and are resistent to nitric acid. But they turn dark when exposed to bright light or chemical reducing agents. This indicates that they consist of silver halogenide. The "argyrophilia" is very useful for the selective staining of the chloride epithelia (Fig. 4a, b and 7). Energy dispersive analysis of x-rays clearly reveals the presence of the elements chlorine and silver in the precipitates (Fig. 6c). Taken together, these date clearly demonstrate that the precipitates consist of silver chloride.

When *A. cyanea* larvae are first placed into 1 mM Na ^{36}Cl solution and then fixed for histochemical chloride precipitation with silver salt-containing fixative, the radioactivity is histoautoradiographically detectable in the precipitates lining the rectal chloride epithelia (Fig. 6e, f). This finding also demonstrates the presence of chloride in the precipitates, and in addition suggests that the chloride ions which are histochemically demonstrable at the rectal chloride epithelia are adsorbed from the hypo-osmotic, external medium ingested through the anus.

Further proof for this conclusion is provided by another type of experiment. Apart from histoautoradiography, radioactivity can also be detected and measured with a liquid scintillation counter, when the histochemical silver chloride precipitates are re-dissolved with 2% Na-thiosulfate after washing of the rectum to remove the water-soluble radioactivity. Counts obtained from the rectum of untreated, mouth-sealed and anus-sealed larvae (Fig. 2c) clearly show that the rectal chloride epithelia adsorb the chloride ions from the external solution ingested with the rectal ventilation via the anus, but not from the urine or from the fluid that may have been ingested orally. Although direct evidence of the exact location of transepithelial ion transport is still lacking, the absorption of chloride from the external medium and the fine structure strongly suggest that the rectal chloride epithelia are responsible for ion uptake into the hemolymph.

ADAPTIVE VARIATION OF EPITHELIAL SIZE

It is well known (WIGGLESWORTH, 1938) that acclimatization of mosquito larvae to different environmental salinities results in different sizes of the anal

chloride epithelium (CE) and the line of autoradiographic silver grains (2) showing identical curvature. (RE: respiratory epithelium; L: rectal lumen; d: phase contrast; e: bright field). 430 x.

papillae which are responsible for osmoregulatory ion absorption in these animals (KOCH, 1938). The rectal chloride epithelia of *A. cyanea* show the same effect. As seen in Figure 7 both chloride epithelia of the gill leaflets are relatively large when the larvae were kept in 0.05 mosm for 1 month, and relatively small when the larvae were kept in 300 mosm for the same period of time.

Planimetric measurements of the chloride epithelia in the mid-region of the rectum of 3 experimental groups (0.05, 5 and 400 mosm) clearly show that the area sizes of the small and large chloride epithelia increase significantly with long-term adaptation to decreasing salinities (Fig. 8a). A corresponding result is obtained when larvae which have been pre-adapted to different salinities are placed in radioactive chloride solution and fixed for histochemical chloride precipitation. Liquid scintillation counting of the re-dissolved silver chloride precipitates in larvae of equal body length reveals increasing chloride adsorption with decreasing salinities of pre-adaptation (Fig. 8c). Provided the amount of chloride adsorbed per unit area is the same, this result also suggests an increase in area size.

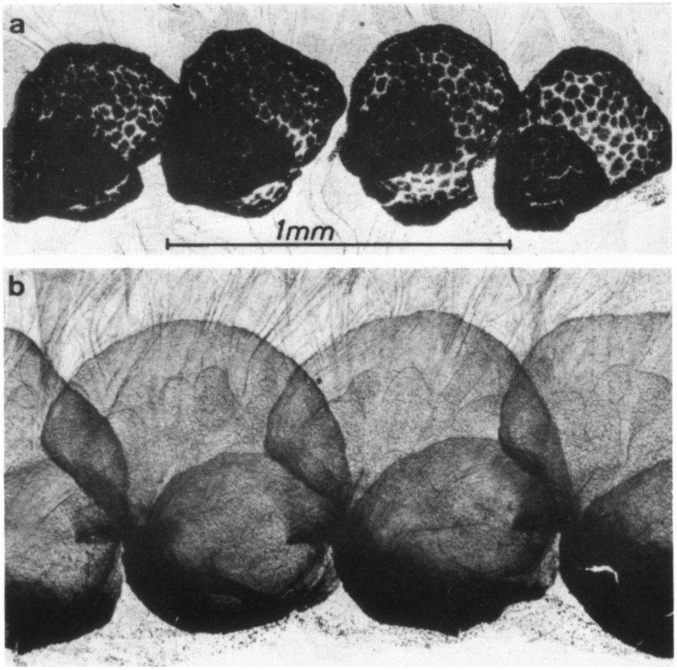

Fig. 7. *Aeshna cyanea*, different sizes of the superimposed large and small chloride epithelia in the mid-region of the rectum after acclimatization of the larvae to different environmental salinities for 1 month: (a) Larva from 300 mosm; – (b) Larva of equal body length from 0.05 mosm external concentration of sea salt. Same kind of preparation as described for Figure 4a, b.

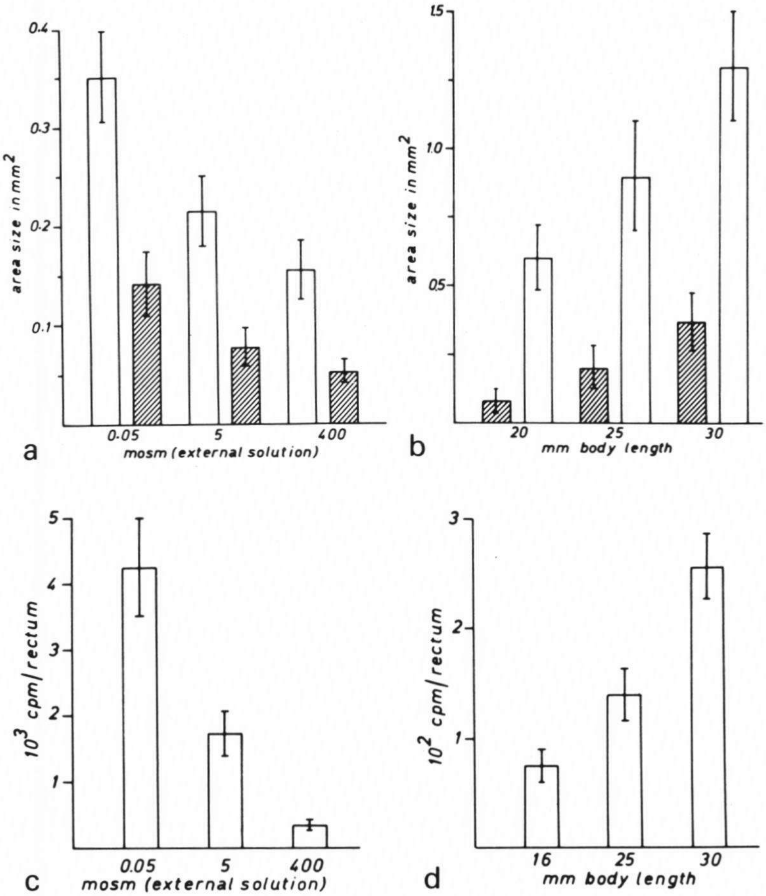

Fig. 8. *Aeshna cyanea*: (a-b) Area size of gill-associated large (open columns) and small (striated columns) chloride epithelia in the mid-region of the rectum. Values obtained with planimetric measurements from preparations like those shown in Figure 7. Mean ± S.D. of 10 to 15 chloride epithelia of 15 larvae: (a) Larvae of equal body lengths (35 mm) after 1 month in different salinities; – (b) Larvae of different body lengths raised at equal salinity (5 mosm). – (c-d) Radioactivity of the re-dissolved silver chloride precipitates in the rectum ($N = 5$): (c) Pre-adaptation for 1 month at different salinities. Pre-adapted larvae of equal body lengths (30 mm) were placed for 2 days in 1 mM Na ^{36}Cl (0.01 μci/ml); – (d) Larvae of different body lengths raised at equal salinity (5 mosm) were placed for 6 hours in 1 mM Na ^{36}Cl (0.01 μci/ml).

The validity of this interpretation is apparent from the fact that different area sizes of the rectal chloride epithelia are also detected with both methods in larvae of different body lengths which were raised under identical external salinities (Fig. 8b, d). Hence, the size of the rectal chloride epithelia appears to be governed by at least two factors, an intrinsic one i.e. the larval stage, and an environmental one i.e. the external salinity. The observed adaptive behavior can be explained according to the following interrelationships: The lower the external salt concentration, the steeper is the gradient between external and internal medium, the greater is the need for ion absorption and the larger is the size of chloride epithelia needed to fulfil the enhanced requirements for absorptive ion transport. This conclusion is supported by the finding that the increase in area size is also paralleled by an increase in the activity of transport ATPases.

TRANSPORT ATPASES OF THE RECTUM

Two ATPases were biochemically studied in homogenates of the rectum of *A. cyanea* larvae, which had been acclimatized to 0.05 mosm and 5 mosm for 1 month. These are the Na^+/K^+-ATPase, which is well known to be part of the sodium transporting system, and HCO_3^--ATPase, which appears to be involved

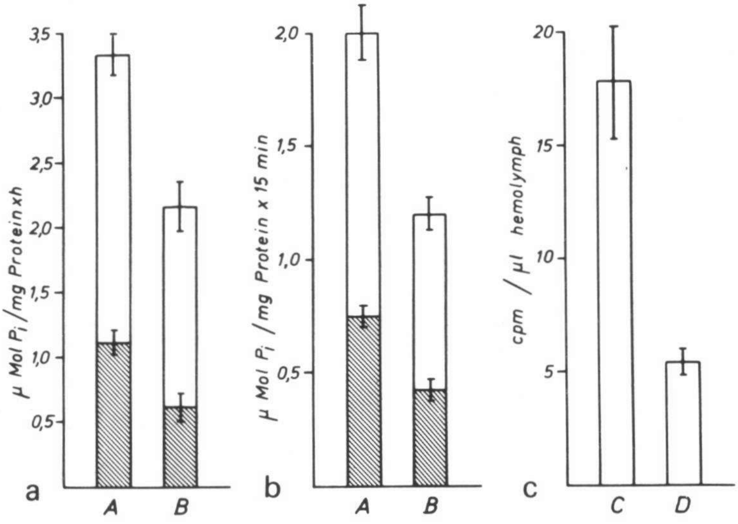

Fig. 9. *Aeshna cyanea*: (a-b) ATPase-activities of the homogenized rectum of larvae pre-adapted for 1 month in different salinities (A: 0.05 mosm; B: 5 mosm), N = 10: (a) Total ATPase (open columns) and Na^+/K^+ stimulated, ouabain-sensitive ATPase activity (striated columns); – (b) Total ATPase (open columns) and HCO_3^--stimulated, thiocyanate-sensitive ATPase activity (striated columns). – (c) Inhibition of chloride uptake into the hemolymph by thiocyanate. Radioactivity in 50µl hemolymph samples taken from larvae (N = 5) after 6 hours in (C) 1 mM Na ^{36}Cl (0.01 µci/ml) plus 20 mM mannitol and (D) 1 mM Na ^{36}Cl (0.01 µci/ml) plus 10 mM NaSCN.

in H^+/HCO_3^--transport (SIMON & KNAUF, 1976) and possibly is also involved in chloride transport (EPSTEIN, MAETZ & DE RENZIS, 1973; DE RENZIS & BORNANCIN, 1977). Both enzymes show significantly higher activities after

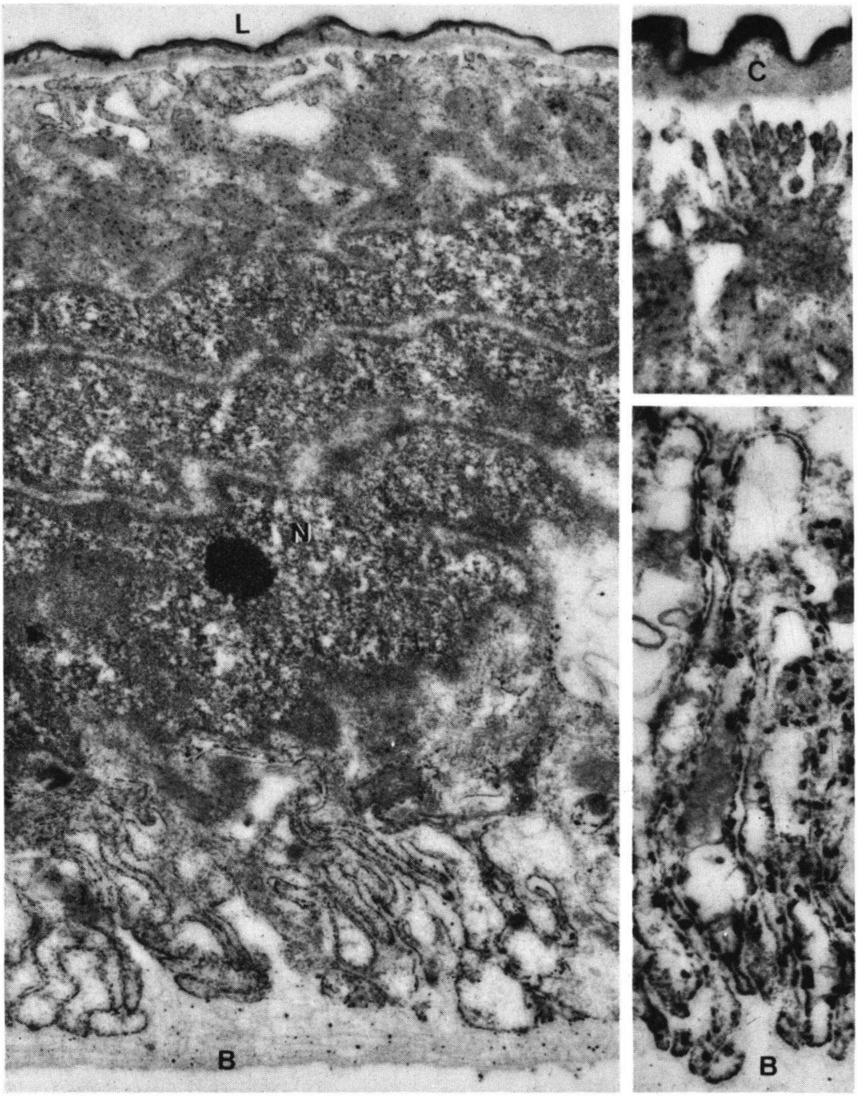

Fig. 10. Histochemical localization of NPPase activity in the rectal chloride epithelium of *Aeshna cyanea*. (L: luminal face and B: basal face of the epithelium; N: nucleus; C: cuticle), 12500 x. The insets show enlarged portions of apical cell membrane folds underneath the cuticle (upper right) and the basal cell membrane infoldings (lower right). 25 000 x.

adaptation to highly dilute medium in comparison to normal freshwater controls (Fig. 9a, b). The observation that thiocyanate, the inhibitor of the HCO_3^--ATPase, also affects chloride uptake, suggests that this enzyme is somehow involved in chloride transport. When 10 mM NaSCN is added to the external labelled chloride solution, chloride absorption into the hemolymph is reduced by about 70% as compared to controls of which the external solution contained 20 mM mannitol for osmotic compensation of the inhibitor (Fig. 9c).

Histochemical investigation of the Na^+/K^+-ATPase with the NPP-method (ERNST, 1972) seems to indicate that the enzyme activity is associated with the plasma membrane, in particular with the baso-lateral cell membrane infoldings (Fig. 10). One critical point of this method is the subjective and difficult evaluation of the decrease in the amount of reaction product in ouabain-treated controls. Therefore, the localization of the Na^+/K^+-ATPase and the HCO_3^--ATPase was jointly studied in subcellular fractions of the rectum.

As shown in Figure 11, there is a close association of both enzymes, their highest activities are found in fraction 3 and 4. Thin sections of the pellets reveal that fraction 3 consists of mitochondria plus membranes, and fraction 4 almost exclusively of membranes (Fig. 12). Since the Na^+/K^+-ATPase is believed to represent a marker enzyme of the plasma membrane, this finding suggests that, in addition to some eventual mitochondrial activity, the HCO_3^--ATPase is also associated with the plasma membrane. However, no conclusion can be drawn from the fractionation studies whether or not the HCO_3^--ATPase is preferentially associated with the plasma membrane of the apical or baso-lateral side of the cells.

The presence of both enzymes in the rectum of dragonfly larvae and their activity dependence from the environmental salinities in long-term acclimatized larvae point to the possible existence of two separate or linked transport mechanisms for active cation and anion absorption.

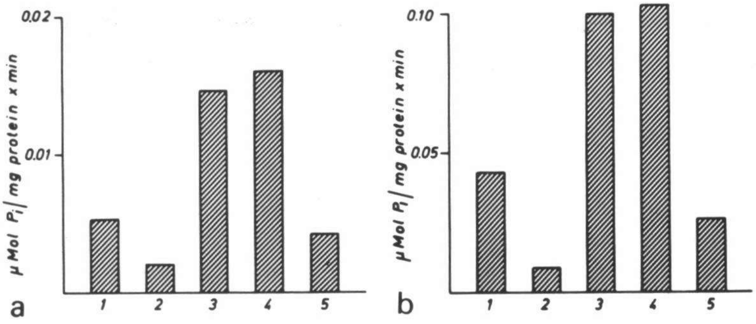

Fig. 11. Activities of (a) Na^+/K^+ - ATPase and (b) HCO_3^- - ATPase in subcellular fractions of the rectum of *Aeshna cyanea* larvae. 1: homogenate; 2: nuclear fraction; 3: fraction containing mitochondria plus membranes; 4: membrane fraction; 5: ribosomal fraction.

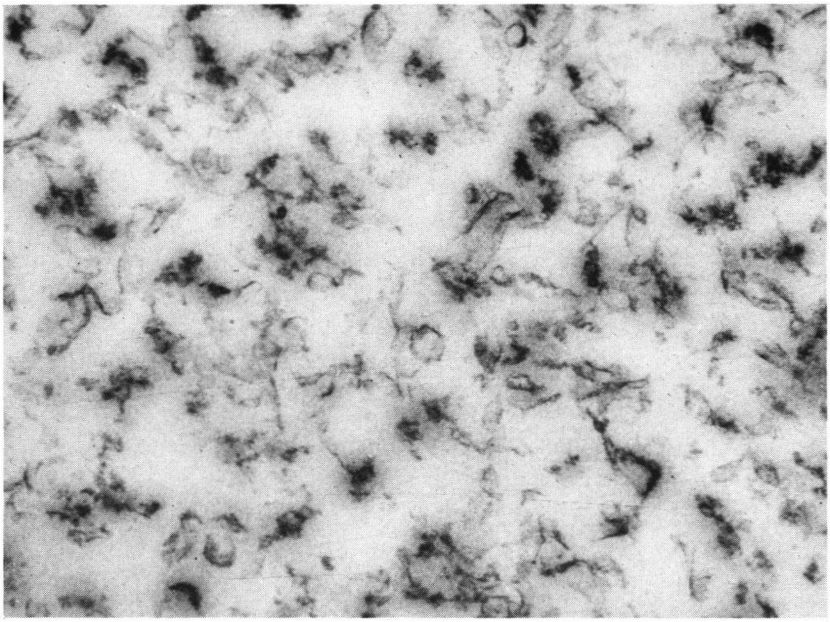

Fig. 12. Electron micrograph of the pellet of fraction no. 4 (membrane fraction), 30 000 x.

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