

## Neuroendocrine Involvement in Osmoregulation in a Freshwater Mollusc, *Lymnaea stagnalis*

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Physiological information on osmoregulation in molluscs has been provided for many species. However, neuroendocrine control of osmoregulation has hardly been studied and cannot, therefore, be dealt with from a comparative point of view. This is mainly due to the fragmentary nature of the histological information on the neuroendocrine system. The freshwater gastropod *Lymnaea stagnalis* is one of the few molluscs in which both the physiology of osmoregulation and the structure of the neuroendocrine system have been studied. This favored the study of neuroendocrine control of osmoregulation. Quantitative analysis at the ultrastructural level indicated that two types of neurosecretory cells are involved. In snails exposed to deionized water, a condition stimulating water elimination and retention and/or uptake of ions, the release of secretory material from the axons of these types was observed. In animals exposed to hypertonic saline, known to reduce water elimination and ion uptake, the release activity declines and an enhanced accumulation of secretory granules occurs. The axons of one type not only end in the main neurohemal zones around the central nervous system but also form a network around the ureter, similar to that found around water and ion transporting epithelia in insects.

After evaluation of physiological data on osmoregulation it is suggested that one type activates water elimination, while the other type stimulates ion-uptake mechanisms. Possible modes of neuroendocrine engagement in osmoregulation are discussed.

### INTRODUCTION

In molluscs the engagement of the neuroendocrine system in the control of osmoregulatory processes is generally assumed. However, in spite of detailed physiological studies regarding water and ion regulation in many molluscan species, physiological evidence for neuroendocrine control of these processes is limited. It has mainly been provided by studies on a pulmonate gastropod, *Lymnaea stagnalis* (Lever *et al.*, 1961), an opisthobranch gastropod, *Aplysia rosea* (Vicente, 1963), and a bivalve, *Crassostrea virginica* (Nagabhushanam, 1964). In these animals extirpation of one or more ganglia of the central nervous system led to swelling of the body, whereas reimplantation or injection of homogenates

of these ganglia resulted in a decrease of the body weight. The production of diuretic factors was assumed to occur in the ganglia concerned. The neurons producing these factors have not been localized so far. In *Aplysia californica* Jahan Parwar *et al.* (1970) recorded changes in the electrical activity of some special neurosecretory (NS-) cells after osmotic stimulation of the osphradium. The functional role of these cells has not been analysed. Additional indications of neuroendocrine engagement in osmoregulation came from histological investigations dealing with osmotically-induced changes in the amount of stainable material present in neuronal cell bodies. These studies, on some bivalve and gastropod species, were recently summarized (Wendelaar Bonga, 1971b) and are all in need of experimental verification.

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The scarcity of data on neuroendocrine

control of osmoregulation in molluscs is closely related to the paucity of information concerning the organization of the neuroendocrine system in these animals, especially in cephalopods and bivalves. This information is in most species studied limited to results obtained with the rather indiscriminative "Gomori" stains or related techniques. This implies that the diversity of the NS-substances, apparent in insects and crustaceans and presumably also present in molluscs, will have largely escaped attention.

In the freshwater snail *Lymnaea stagnalis*, however, the histochemical and ultrastructural aspects of the neuroendocrine system have been studied to a larger extent (e.g., Joosse, 1964; Boer *et al.*, 1968; Wendelaar Bonga, 1970). Furthermore, a series of studies has been presented dealing with the physiology of osmoregulation in this species (e.g., Van Aardt, 1968; Greenaway, 1970, 1971). Indications for neuroendocrine involvement in osmoregulation have also been reported (Hekstra and Lever, 1960; Lever *et al.*, 1961; Lever and Joosse, 1961). Since in other molluscs the available data on either physiological or neuroendocrine aspects of osmoregulation are less complete, this study will be largely confined to *L. stagnalis*. Since the osmoregulatory processes in this snail appeared to conform in detail to the patterns generally encountered in freshwater molluscs, its mechanisms of neuroendocrine control may be of wider significance.

The NS-system of *L. stagnalis* is complex and includes at least 9 types of neurons. These neurons have several characteristics in common with the established peptidergic neuroendocrine systems of vertebrates, insects, and crustaceans: affinity for protein stains, the presence of elementary granules, and nonsynaptic axon terminals in neurohemal zones. These zones are very extensive in *L. stagnalis*, and include the periphery of the intercerebral commissure and of a number of connectives and nerves, and the loose connective tissue around the central nervous system (CNS) (Wendelaar Bonga, 1970).

Freshwater molluscs, like *L. stagnalis*,

maintain a high osmotic gradient between blood and environment. This gradient causes an inward flow of water and an outward diffusion of solutes. The processes for maintaining a steady state generally involve the production of large volumes of a hypotonic urine and an active uptake of ions, as has been established for several molluscs including *L. stagnalis*. To study the relation between NS-cells and these processes, snails were exposed to media of different osmotic and ionic composition, viz., tap water, deionized water, and 0.1 M NaCl solution in deionized water. From data of Van Aardt (1968) and Greenaway (1970) for *L. stagnalis* it was concluded that deionized water will activate water elimination and the ion-uptake mechanisms, whereas saline will lead to a suppression of both processes. Consequently, the presumed neuroendocrine systems engaged in their control should be stimulated in deionized water and inactivated in saline.

Attention was paid to a cell type occurring in both pleural ganglia, the Dark Green Cells (DGC). It was observed that the DGC were activated in deionized water, as was reported before (Wendelaar Bonga, 1971b). During two weeks of exposure a statistically significant increase was noted in the extent of the granular endoplasmic reticulum, in the activity of the Golgi complex, and in the release activity in the neurohemal zones. Saline exposure suppressed the secretory processes and led to a general cytoplasmic regression.

Indications were obtained that another NS-cell type, the Yellow Cells (YC) reacts to osmotic variations of the medium. These neurons occur in small groups in both parietal ganglia and in the single visceral ganglion (Fig. 1). Their neurohemal areas are primarily located in the periphery of the proximal parts of the nerves originating from these ganglia and of the connectives between these ganglia. The YC also attracted attention for another reason: traces of their secretory material, staining yellow with the Alcian Blue/Alcian Yellow technique (Wendelaar Bonga, 1970), were observed in the distal parts of some

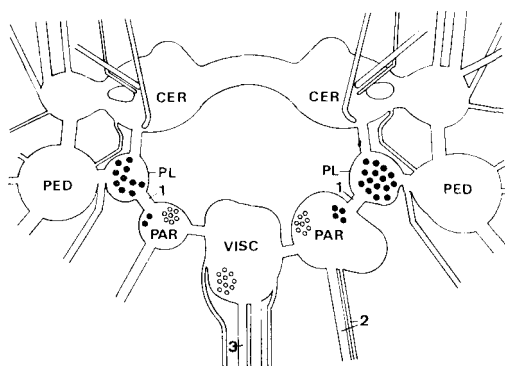


FIG. 1. Diagram of the central nervous system of *Lymnaea stagnalis*. The location of the DGC (dots) and of the YC (circles) is indicated. CER: cerebral ganglia; PL: pleural ganglia; PAR: parietal ganglia; VISC: visceral ganglion; PED: pedal ganglia; 1: pleuro-parietal connectives; 2: right pallial nerves; 3: anal nerve.

of the nerves mentioned, the right pallial nerves and the anal nerve, as far as their effector areas, in the kidney region.

In this study the effects of osmotic changes in the environment on the release activity of the YC, and, for comparison, of three types of NS-neurons, including the DGC, were analysed in the central neurohemal areas. Furthermore, the yellow-staining axons in the kidney were investigated to establish whether their secretory material is released, like neurotransmitters, in synaptic contacts, or, like neurohormones, in free nerve endings. Finally, the release activity in these axons was studied under changed osmotic conditions.

## RESULTS

Groups of 5 adult animals, reared in tap water, were exposed for 24 hr, at 20°C, to deionized water or to NaCl solutions in deionized water. The osmolality of the blood of these snails was determined (Fig. 2). It appears that *L. stagnalis*, like all freshwater invertebrates, regulates only hyperosmotically. The snails maintain an osmotic difference between the blood and the outer medium over a wide range of salinities, up to 0.1 M NaCl. At higher concentrations the blood is slightly hypertonic.

For analysing the changes in the axon terminals the groups exposed to deionized

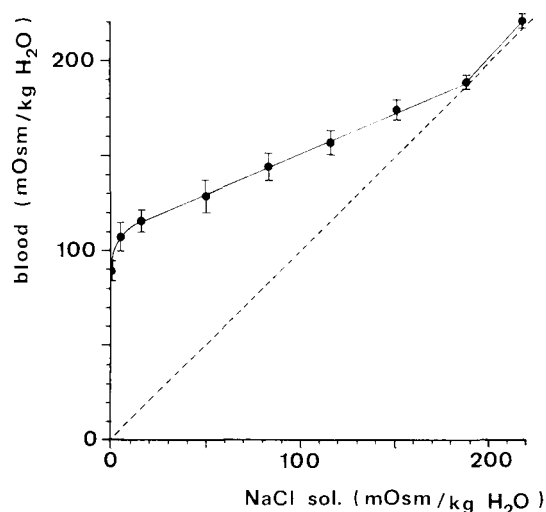


FIG. 2. The osmolality of the blood of the cephalic sinus (steady state) of groups of 5 specimens of *L. stagnalis* (mean values per group,  $\pm$ SE), exposed for 24 hr at 20°C to deionized water (0.5 mOsm) or to different NaCl solutions. Diagonal: isosmotic line.

water (0.5 mOsm) and to 0.1 M NaCl (187 mOsm) were studied at the ultrastructural level, as were 5 control animals kept in tap water (15 mOsm). A detailed account of the materials and techniques has been presented before (Wendelaar Bonga, 1971b).

### 1. The Central Neurohemal Areas

The accumulation of the NS-material in the axon terminals in the central neurohemal zones was quantified by counting the number of axon profiles containing the elementary granules of the YC, in 3 cross sections per animal of the anal nerve. These sections were cut at distances of about 100  $\mu$  from each other, near the origin of the nerve. The release activity was estimated in the same sections by counting the number of axon profiles showing release phenomena (clusters of clear vesicles and omega-shaped indentations of the axonal membrane, cf., Wendelaar Bonga, 1971a,b). In addition, accumulation and release were determined, in the same sections, of two other types of NS-axons terminating in the anal nerve, which originated from the Light Yellow Cells (LYC) and from the Yellow Green Cells (YGC). The axon terminals of the DGC were examined in 3

cross sections per animal of the right pleuro-parietal connective.

As appears from Fig. 3, exposure to deionized water results in a decrease in the number of granule-filled axon endings of both the DGC and the YC, and an increase in axons showing release phenomena (Fig. 4). An increase of empty and nearly empty axon terminals was observed (Fig. 5). In saline the reverse occurs: an accumulation of secretory granules and a reduction of release activity. The changes are statistically significant with Student's *t*-test ( $p < .05$ ). The axon terminals of the LYC and the YGC did not show obvious changes in either the accumulation or the release of their secretory material. These types apparently do not react to variations in the osmotic conditions of the snails, a conclusion which was reached earlier on the basis of a quantitative analysis of the cell bodies of these cell types after 14 days of exposure to the same medium (Wendelaar Bonga, 1971b).

## 2. The Axons Terminating in the Kidney

The kidney, an elongated tubule lined by a highly folded epithelium, is composed of a kidney sac and a primary ureter. The

ureter cells show the general ultrastructural features of epithelia involved in water and ion transport, viz., extensive basal infoldings of the cell membrane, associated with many mitochondria (Wendelaar Bonga and Boer, 1969). The kidney is profusely provided with blood lacunae. The epithelium is separated from the blood by a thin (1–5  $\mu$ ) layer of connective tissue. In the ureter a fine network of small nerves and single axons is found in this layer. Most of these axons contain elementary granules which are similar in size and appearance to those of the YC (Fig. 6). No synaptic contacts between these axons and kidney cells or connective tissue elements were observed. However, release phenomena were frequently observed in axonal areas facing the blood or the connective tissue matrix. Thus, the secretory material of these axons is released in a way characteristic for neuroendocrine substances.

The effects on the axons surrounding the ureter of exposure of snails to deionized water and to 0.1 *M* NaCl were analysed. The degree of accumulation of the secretory material was determined by counting the total number of axon profiles containing the YC-type of elementary granules in

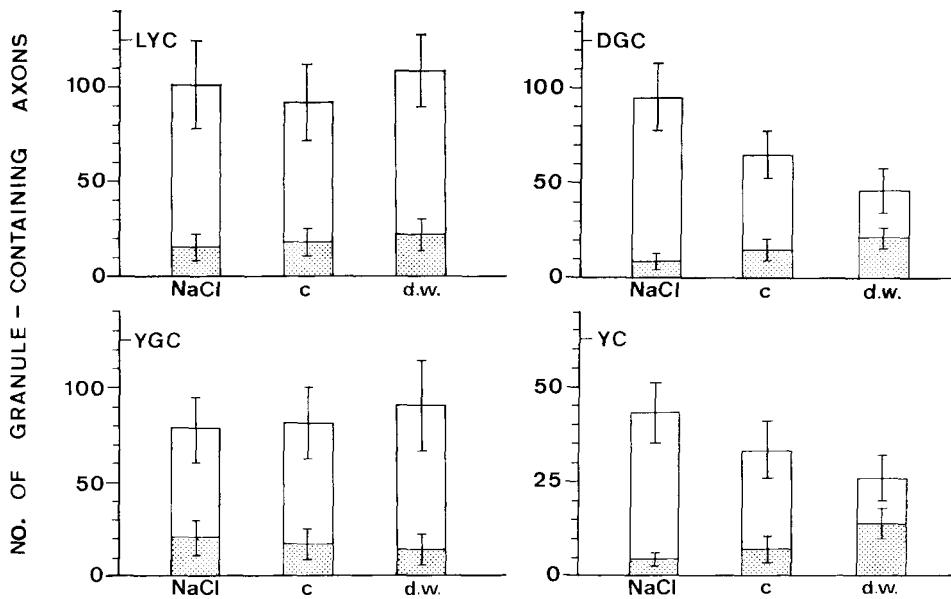


FIG. 3. The number of granule-containing axon terminals (total length of bars) and of the fraction of these terminals showing release phenomena (stippled bars) of 4 types of neurosecretory cells. Each bar represents the mean value ( $\pm$ SE) of 5 snails. c: controls, kept in tap water; NaCl, and d.w.: snails exposed to 0.1 *M* NaCl and to deionized water, respectively, for 24 hr.

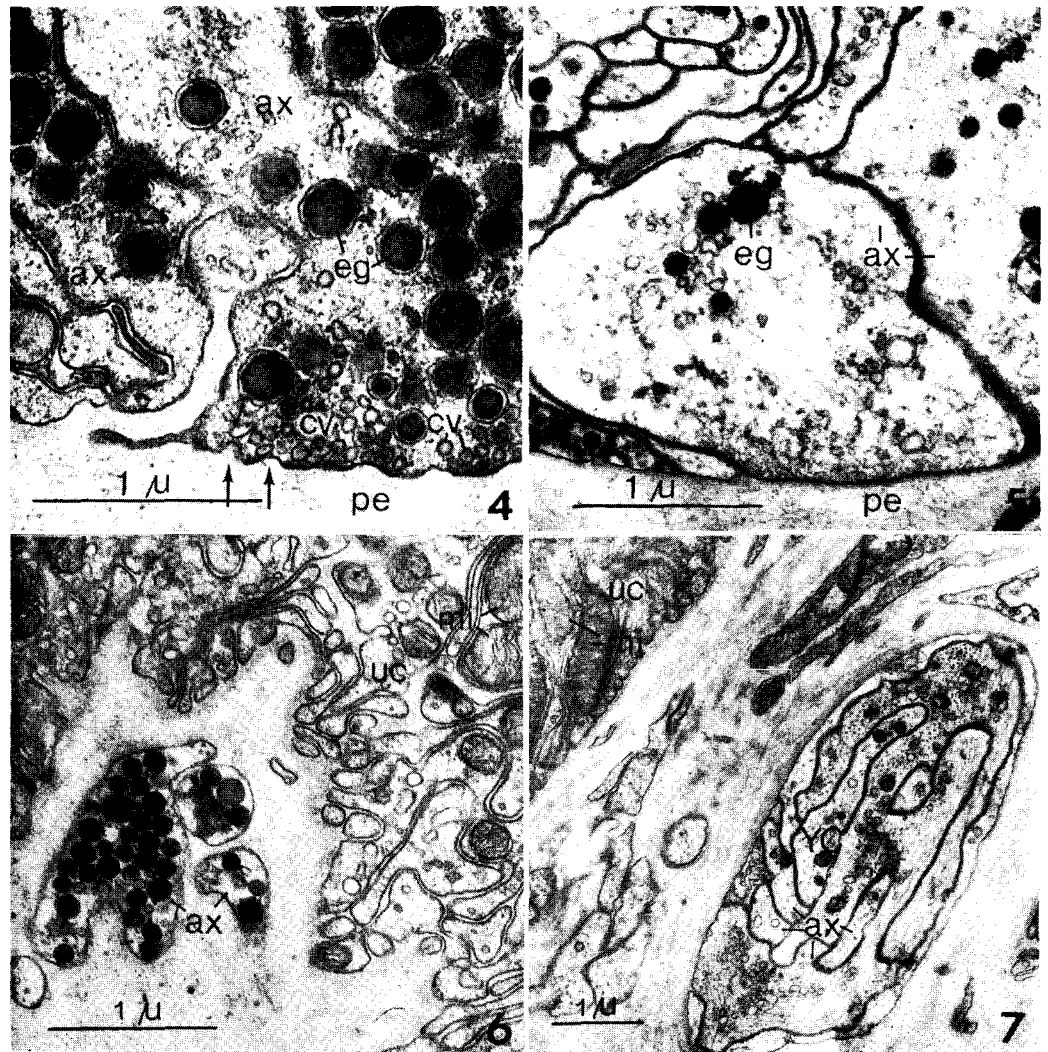


FIG. 4 and 5: Axon terminals of the DGC (ax) in the periphery of the right pleuro-parietal connective (24 hr in deionized water); pe: perineurium; eg: elementary granules. Fig. 4: Terminal showing clear vesicles (cv) and membrane indentations (arrows), indicative of release activity. Fig. 5: Terminals almost devoid of elementary granules.

FIG. 6 and 7: Axons in the connective tissue layer around the ureter wall. The ureter cells (uc) show basal infoldings associated with mitochondria (mi). Fig. 6: Control. Axons containing elementary granules of the YC-type. Fig. 7: Deionized water, 24 hr. Small nerve showing many axons (ax) which, except for one axon (YC), are devoid of elementary granules.

cross sections of the ureter. Three sub-epithelial zones (each with a length of  $1000\ \mu$ ) per animal—one cut from the middle, the other two from each end of the ureter—were sampled. The release activity was assessed by counting the number of axons showing release phenomena.

Figure 8 shows that deionized water causes an increased release of secretory

material. The number of granule-containing axon terminals was reduced, whereas the number of these axons showing release phenomena increased. Many empty, or nearly empty, axons were found (Fig. 7). In saline the release activity was reduced, which led to an accumulation of elementary granules: the number of axon profiles containing elementary granules increased.

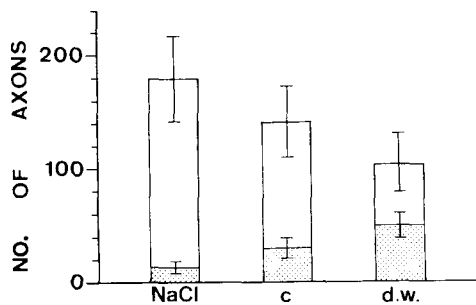


FIG. 8: Number of axon profiles containing elementary granules of the YC-type (total length of bars) and of the fraction of these profiles showing release phenomena (stippled bars) present in the subepithelial connective tissue layer of the ureter. Each bar represents mean values ( $\pm$ SE) of 5 animals. c: controls, kept in tap water; NaCl, and d.w.: snails exposed to 0.1 M NaCl and to deionized water, respectively, for 24 hr.

It is concluded that the changes (all statistically significant,  $p < .05$ ) occurring in the axons in the ureter wall in both media are similar to those observed in the axon endings of the YC in the central neurohemal areas. This is a further indication that the axons in the ureter originate from the YC.

#### DISCUSSION

##### 1. Morphological Aspects of the DGC and of the YC

In the absence of adequate bioassay techniques, the quantitative analysis of NS-cells at the ultrastructural level appears to be an effective way to study changes in the release activity. The morphological phenomena accompanying release have been studied in a large number of animal species. Quantal release via exocytosis, as found in *L. stagnalis* (Wendelaar Bonga, 1970, 1971a), has been reported for many, although not all, of them. The presence of clusters of clear vesicles is generally agreed to be indicative of release (Scharrer, 1969). Therefore, an increase in these phenomena, as established in the present experiments in the axons of the DGC and the YC after exposure to deionized water, points to an increased transport of secretory material towards the body fluid.

The secretory material in the axonal network in the ureter, apparently originating from the YC, is released in free nerve endings. Indications were found that in *L. stagnalis* the YC are not the only type of NS-neurons which release their material in the central neurohemal areas as well as in peripheral parts of the nervous system. Around the vagina, axons of the LYC were found. Peripheral neurosecretion has been described in other molluscs (Cottrell and Osborne, 1969) and in many insect species (Maddrell, 1969). Of particular interest is the observation of NS-axons ending non-synaptically underneath the ileac and rectal pads in some insects (e.g., Jariol and Scudder, 1970); these pads consist of epithelia specialized, like the ureter of *L. stagnalis*, for transport of water and ions. This type of innervation, also found in corpora allata of insects and in the teleost adenohypophysis, may provide a high concentration of the mediator in its effector area. The range of action of the substance concerned is intermediate between that of hormones, transported by the blood, and that of neurotransmitters, which release their material into a synaptic cleft (Scharrer, 1970).

##### 2. Neuroendocrine Involvement in Osmoregulation

In normal conditions the inward water flow generated by the osmotic difference between inner and outer media in freshwater animals will be counteracted by the hydrostatic pressure exerted by the body, and by renal water excretion. In the steady state the total water influx will equal the water elimination by the kidney. In gastropods the urine formation is probably accomplished by ultrafiltration across the heart wall into the pericardial space. The resulting prourine is transported via the reno-pericardial duct towards the kidney. (Potts, 1967). Some experimental evidence for this process in *L. stagnalis* was presented by Van Aardt (1968). He found that the urine production is high, amounting to 20% of the blood volume (9% of the body weight) per hour. This high rate

is understandable as the osmolality of the blood in *L. stagnalis* is the highest found in freshwater molluscs, and the rate of urine formation is generally found to be proportional to the osmotic difference between inner and outer media (cf. Chaisemartin *et al.*, 1970).

High salt losses are known to occur in freshwater animals, due to outward diffusion and to urine excretion. Although hypotonic to the blood (Van Aardt, 1968), urine is highly hypertonic to the medium. In *L. stagnalis* losses are compensated by active ion transport by the skin (Greenaway, 1970, 1971), the ureter (Van Aardt, 1968), and possibly the gastrointestinal tract. The role of the last organ in osmoregulation in molluscs is not known. The occurrence of Na<sup>+</sup>- and Cl<sup>-</sup>-uptake mechanisms, widespread in freshwater organisms (Kirschner, 1970), is indicated for *L. stagnalis*. Greenaway (1970, 1971) concluded that independent transport mechanisms were present for these ions and also for Ca<sup>2+</sup>. The uptake rates for both Na<sup>+</sup> and Ca<sup>2+</sup> showed Michaelis-Menten kinetics.

The main hormonal systems in *L. stagnalis* seem to be engaged in the control of urine formation, of the permeability of the skin, and of the ion uptake mechanisms. Some experimental indications of endocrine involvement in these processes in *L. stagnalis* can be deduced from available data. Exposure to deionized water, known to result in an increased water uptake, does not lead to a rise in the body weight: during the first 24 hr a slight decrease even occurred while the snails had a shrunken appearance (Wendelaar Bonga, 1971b). A similar observation was made by Greenaway (1970) who further noticed that the Na<sup>+</sup>-concentration of the blood of snails kept in deionized water was higher than might have been expected on the basis of measured Na<sup>+</sup>-losses. These observations may imply that in deionized water the blood volume is reduced, by forced water elimination and/or by reduction of the permeability of the skin to water, to compensate for the loss of solutes from the blood. This points to the presence of an endocrine reflex mechanism for control of

water balance. Furthermore, the initial net loss of Na<sup>+</sup> and Ca<sup>2+</sup> from snails in deionized water is followed by a net uptake of these ions (Greenaway, 1970, 1971). The rate of ion uptake appeared to be related to the internal concentration of the ions concerned. A similar relation is known for other freshwater animals, e.g., the gastropod *Viviparus viviparus* (Little, 1965) and the earthworm *Lumbricus terrestris* (Dietz and Alvarado, 1970), and also suggests an endocrine reflex mechanism for the maintenance of the ion balance, like that occurring in vertebrates.

In *L. stagnalis* two types of NS-neurons, the DGC and the YC, appeared to be activated in snails placed in deionized water. Studies of Hekstra and Lever (1960) and of Lever *et al.* (1961) support the hypothesis that the DGC stimulate diuresis. Removal of the pleural ganglia, which contain most of the DGC, led to a considerable swelling of the snails. Injection of homogenates of these ganglia in intact animals reduced body weight. The authors suggested the presence of a diuretic factor in these ganglia. This supposition has been corroborated by results obtained in a related species, *Lymnaea limosa*. Cauterization of the pleural ganglia caused, in addition to an increase in body weight, a reduction in urine formation. No effect on the sodium fluxes was noted (Chaisemartin, 1968). Urine measurements under similar conditions in *L. stagnalis* are lacking so far, but it may be assumed that the results would be comparable. Although definite proof is needed, it is therefore indicated that the substance of the DGC accounts for the diuretic factor postulated by Lever and coworkers. This substance might act by influencing the hydrostatic pressure of the body fluid, the force accounting for ultrafiltration. An increase of this pressure may result from stimulation of the tonicity of the musculature of the body wall and/or of the blood vessels, or from stimulation of the heart. The latter possibility was proposed by Chaisemartin (1968) who found in *L. limosa* a decreased heart rate after destruction of the pleural ganglia. In *L. stagnalis*, however, the heart rate did not

change during exposure to deionized water.

With regard to osmoregulation, no research has so far been performed on the paired parietal ganglia and the single visceral ganglion of *L. stagnalis*, the ganglia containing the cell bodies of the YC. In *L. limosa*, cauterization of the parietal ganglia resulted in a decreased turnover of  $\text{Na}^+$ -ions, as was demonstrated by Chaisemartin (1968) with radioisotopes. A neuroendocrine substance produced in these ganglia and influencing the ion transport mechanisms was postulated by this author. The present data suggest that the product of the YC stimulates these mechanisms in *L. stagnalis*. The ion-uptake mechanisms located in the body wall and in the ureter may be controlled by the same substance (Little, 1965). The striking distribution of the axon terminals of the YC, which occur in the central neurohemal zones and around the ureter, may be the morphological expression of such a dual function.

At the salinity used in our experiment (0.1 M) the blood is almost isotonic with the medium. Thus, the osmotic inflow of water will be minimal and the rate of urine formation will be low. A decreased elimination of water was indeed observed at concentrations of 0.1 M NaCl and higher (Van Aardt, 1968). An initial shrinkage of the animals, when placed in hypertonic solution, was expected; however, swelling was observed instead (Wendelaar Bonga, 1971b). This response may indicate that blood volume is increased. Obviously water elimination is further reduced than is needed for maintaining a constant body volume. This may be a reaction to compensate for the inward flow of ions. An endocrine reflex mechanism is suggested by this phenomenon. Reduced activity of the hormonal systems involved in the stimulation of water elimination and the uptake of ions, and activation of possible factors with an antagonistic function, were expected. The observed decrease of the release activity of the DGC and of the YC in saline adds further evidence to the hypothesis that these cell types are involved in stimulating water elimination and ion

uptake. Evidence for the presence of antagonistic neuroendocrine factors—substances stimulating loss of salts and limiting water elimination—is almost absent in molluscs. The observation of Joosse and Lever (1961) that some NS-cells in the cerebral ganglia of *L. stagnalis* are depleted of secretory material is the only histological indication to date. Moreover, no clear physiological indication of anti-diuresis has been presented. Observations of hypotonic regulation in molluscs are not known. In NaCl concentrations higher than 0.1 M, the blood of *L. stagnalis* is slightly hypertonic. A similar hypertonicity is known for other freshwater invertebrates exposed to higher salinities, and from marine animals under natural conditions and is partly due to organic compounds in the blood (Pierce, 1971). When the blood is nearly isotonic the inorganic ion compositions of blood and medium are by no means similar. Important differences, especially in  $\text{K}^+$  and  $\text{Ca}^{2+}$ , are known. Thus, the capability of molluscs to hyporegulate the content of one or more ions cannot be excluded. It has been claimed by Dietz and Alvarado (1970) that the  $\text{Cl}^-$ -concentration of an earthworm, when kept in saline, is hyporegulated. Differences in ionic composition between blood and medium may, however, also have a physical basis, and represent electrochemical or Gibbs-Donnan effects. The high  $\text{K}^+$ -content in the blood of marine molluscs is accounted for by a Donnan equilibrium (Pierce, 1971). More information is needed in this respect.

A lack of data also exists with respect to the control of osmoregulation at the cellular level. Cellular osmoregulation, involving in part inorganic ions and in part free amino acids, is essential to keep the cell volume constant (Florkin and Schoffeniels, 1969), and has been studied in some marine molluscs as well as in freshwater species (Pierce, 1971). Information is scarce, however, as to the effects of cellular osmoregulation on the composition and pH of the blood in animals under changing osmotic conditions. Such effects may be important. Chaisemartin *et al.* (1970) noted a selective accumulation of  $\text{Na}^+$  in



the tissues of *L. limosa* exposed to high salinities.

It is concluded that additional information on some details of the process of osmoregulation in molluscs is needed. However, the main lack of data concerns the structure of the neuroendocrine system, which hampers endocrinological experimentation in nearly all species. This explains why neuroendocrine control mechanisms in molluscs are in general so badly understood and why these mechanisms cannot yet be dealt with from a comparative point of view.

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#### DISCUSSION

GOLDING: What criteria did you use to distinguish between active and inactive neurosecretory terminals, that is, between those terminals which were releasing as opposed to those that were not.

BONGA: Axon terminals in the release stage were distinguished by the presence of clusters of clear vesicles and of omega-shaped indentations of the axonal membrane. In adequately fixed material (fixation is very critical for the preservation of exocytosis), both features were usually found together. In the absence of membrane indentations the terminals were only considered in the release stage when clusters of many clear vesicles were present close to the outer axonal membrane.