OSMOTICALLY INDUCED CHANGES IN THE ACTIVITY OF NEUROSECRETORY CELLS LOCATED IN THE PLEURAL GANGLIA OF THE FRESH WATER SNAIL LYMNAEA STAGNALIS (L.), STUDIED BY QUANTITATIVE ELECTRON MICROSCOPY

by

S. E. WENDELAAR BONGA
(Department of Biology, Free University, Amsterdam, The Netherlands)

CONTENTS

I. Introduction....................................................................................................................... 127
II. Materials and Methods............................................................................................... 129
III. Observations.................................................................................................................. 131
   1. The Dark Green Cells ............................................................................................... 131
      a. The Cell Bodies of the Dark Green Cells ................................................132
          a. Controls......................................................................................................... 133
          b. Snails exposed to De-ionized Water .........................................................136
          c. Snails exposed to 0.1 M Sodium Chloride ........................................... 139
      B. The Axons of the Dark Green Cells .................................................................. 143
          a. Controls......................................................................................................... 144
          b. Snails exposed to De-ionized Water .........................................................145
          c. Snails exposed to 0.1 M Sodium Chloride ........................................... 146
   2. Other types of Neurosecretory Cells ...................................................................... 147
IV. Discussion....................................................................................................................... 147
   1. Methods....................................................................................................................... 147
   2. The Cell Bodies of the Dark Green Cells .........................................................148
   3. The Axons of the Dark Green Cells .................................................................. 151
   4. Functional significance of the Dark Green Cells ...............................................152
V. Summary............................................................................................................................ 154
Acknowledgements.........................................................................................................155
References....................................................................................................................... 155

I. INTRODUCTION

In experiments involving the extirpation or destruction of parts of the central nervous system, often followed by injection of homogenates or by reimplantation of these tissues, the presence of osmotically active factors of supposed neuroendocrine origin has been demonstrated in many invertebrate groups, e.g., in annelids (KAMEMOTO, 1964; LECHENNAULT, 1965; DRAWERT, 1968), insects (GIRARDIE, 1966, 1970) and molluscs (HEKSTRA & LEVER, 1960; LEVER et al., 1961; NAGABHUSANAM, 1964). Variations in the amount of stainable material of
neurosecretory neurones induced by exposing animals to media of different ionic and osmotic concentrations have been reported for numerous species (cf. Drawert, 1968), presenting further indications that, as in vertebrates, the neuroendocrine system in invertebrates is involved in osmoregulation.

Hekstra & Lever (1960), while investigating the pond snail Lymnaea stagnalis, observed an increase in body weight, due to water uptake, after extirpation of the pleural ganglia. Extirpation of the parietal ganglia had a moderate effect, as had sectioning of the pleural connectives. In further experiments (Lever et al., 1961) implantation of pleural ganglia after pleuralectomy proved to annihilate the swelling. Injection of homogenates of the pleural ganglia in intact animals resulted in a decrease of the body weight during the first hours. The authors concluded that the pleural ganglia contain a factor which exerts a diuretic influence. Since this factor is apparently able to act via the blood stream, it was assumed to be an endocrine substance.

It was found, in a histochemical and morphological study of the neuroendocrine system of Lymnaea stagnalis (Wendelaar Bonga, 1970a,b), that the pleural ganglia contain a specific group of secretory neurones. These were called the Dark Green Cells (DGC) on the basis of their reaction with the Alcian Blue/Alcian Yellow staining method. The extensive neurohaemal areas of these cells are primarily located in the pleural connectives. As the experiments of Lever and coworkers involved the removal of these neurones as well as a destruction of their main neurohaemal zone, the observed diuretic effects of the pleural ganglia may be due to the secretory products of the DGC. For this reason the relation between these neurones and the water balance of the snail was studied.

The osmoregulatory capacities of Lymnaea stagnalis are impressive (Van Aardt, 1968; Greenaway, 1970), as is common for fresh water animals. Its blood osmotic pressure is the highest (110–120 mOsm/kg H₂O) observed so far in fresh water molluscs. L. stagnalis can maintain an osmotic gradient between the blood and the external medium in de-ionized water, as well as in saline solutions up to 0.1 M NaCl. At the latter concentration the blood appears to be almost isotonic with the medium, as was found during the present investigations. Under natural conditions the species stands a wide range of salinities, as is indicated by its occurrence in fresh water as well as in the Baltic Sea, at concentrations up to 0.1 M NaCl (cf. Boettger, 1944).

The osmotic gradient between blood and medium leads to an osmotic water uptake across the body wall. This inward flow is compensated by the excretion of urine. As the rate of water uptake and consequently the rate of diuresis, is related to the height of the osmotic
gradient (Van Aardt, 1968), both processes will be stimulated when the animals are exposed to de-ionized water. Exposure to saline solutions, on the other hand, will reduce the urine production, as was found by Van Aardt. When diuresis is under endocrine control, the first treatment will lead to enforced synthesis and release of the diuretic factor, the latter to suppression of these secretory processes. Thus, if the DGC are involved in diuresis, it may be assumed that exposure to de-ionized water will activate these neurones, whereas exposure to saline will induce hypoactivity. Therefore, experiments were carried out to investigate whether the secretory processes of the DGC are influenced by exposing specimens of Lymnaea stagnalis, normally kept in tap water, to these media.

As in other invertebrates, osmotically induced changes in neurosecretory cells in molluscs have so far only been examined at the light microscope level, usually after staining with the Gomori methods for neurosecretion (Boddingius, 1960; Lever & Joosse, 1961; Jungstand, 1962; Lubet & Pujol, 1963; Nagabhushanam, 1964; Andrews, 1968). Since it is difficult to interpret the results obtained by these stains in terms of changing neurosecretory activity of the cells concerned (Highnam, 1965; Mordue, 1967), additional evidence is needed to elucidate the effects of osmotic treatments on the secretory processes. In a study on diurnal secretory rhythmicity in neurones of Lymnaea stagnalis, it appeared that the changes in the rates of synthesis, transport and release of secretory material can be described by making a quantitative analysis of the neurones at the ultrastructural level (Wendelaar Bonga, 1971). Accordingly, to examine the effects of de-ionized water and saline on the DGC, quantitative electron microscopy was applied.

II. MATERIALS AND METHODS

Sexually mature specimens of Lymnaea stagnalis (L.), about 6 months old, with a shell height of 30–32 mm, were obtained from stock reared in the laboratory in tanks with continuous water change under conditions described by Van der Steen et al. (1969). During an acclimatization period of three weeks and during the experiments, the animals were kept individually in jars containing 500 ml of tap water, which was changed every third day and had a temperature of 20 ± 1°C. A daily photoperiod of 12 hours (from 7 a.m. till 7 p.m.) was applied.

For the main experiments three groups of about 70 snails each were used. One group was placed in de-ionized water (0.1–0.5 mOsm/kg H₂O). Another group was placed in 0.1 M NaCl in de-ionized water (187 mOsm/kg H₂O). From each group 15 snails were fixed on day 1,
8, and 15, respectively. After 15 days of exposure the remaining animals were replaced into tap water, and a week later 15 snails of both groups were killed (at day 22 of the experiments). The snails of the third group (controls) were continuously kept in tap water. Before the experiments (at day 0) 15 of them were killed. Mortality was rather low in all groups. During the experiments 24 snails died: 7 of the first group, 13 of the second group, and 4 of the controls. Lettuce was supplied every third day for a period of 24 hours, prior to water change. The food consumption was determined by weighing the lettuce before and after the 24 hour period. The increase of the osmolality of the media due to the presence of food and snails never exceeded 3 mOsm.

After decapitation of the snails the central nervous systems and the proximal parts of the nerves were excised. Out of each group of nervous systems 3 were prepared for light microscopy. After paraffin embedding and sectioning these were stained with the Alcian Blue/Alcian Yellow staining method (Wendelaar Bonga, 1970a). The remaining nervous systems were prepared for electron microscopy; 9 of each group were fixed for 2 hours in a Veronal buffered (pH 7.4) mixture of glutaraldehyde (0.8%) and osmium tetroxide (1.0%) at 1°C. After dehydration the right pleural and parietal ganglia were excised, the right pleuro-parietal connective being left intact, and embedded in Epon 812. For further examination 5 of these pairs of ganglia were selected. As cross sections of the pleuro-parietal connective had to be sampled, the criterion for selection was the diameter of this connective (Ø: 110-125 μ). This diameter was determined in 2 μ thick Epon sections by use of an eyepiece micrometer, in a phase contrast microscope.

To study the Dark Green Cells a series of ultrathin sections was cut of the pleural ganglia, at distances of 5-10 μ. Furthermore, in order to examine the axon terminals of these neurones, 3 cross sections were cut from each right pleuro-parietal connective.

The nervous systems of 3 snails of each group, killed at day 15 of the experiments, were prepared for the demonstration of acid-phosphatase activity. The tissue was prefixed in 2% glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.2) for 30 min. After rinsing in 1.5% sucrose in the same buffer, incubation followed in a medium prepared after Barka & Anderson (1962) at 37 °C (pH 5.2) for 30 min. The tissue was rinsed in acetate buffer (pH 5.2) and fixed in a 1% acetate buffered (pH 7.2) solution of osmium tetroxide containing 10% sucrose. Controls were incubated after adding 0.01 M NaF, or after omitting the substrate, β-glycerophosphate.

The ultrathin sections, cut on a Reichert Ultramicrotome, were stained with lead citrate and examined in a Zeiss EM 9A electron microscope.
The mean volume of the cytoplasm of the cell bodies of the DGC was determined in light microscope sections according to the method of Siew (1965). For each determination 60 neurones were measured in 3 animals (20 neurones per animal). The mean values (± S.E.) are indicated in the text.

The osmolalities of the blood and of the media were determined on samples of 0.2 and 2.0 ml, respectively, in an Advanced Osmometer Model 3A. Blood samples were obtained from the cephalic sinuses of snails (10 per group) treated similarly to the experimental groups.

III. OBSERVATIONS

1. THE DARK GREEN CELLS

The DGC are mainly found in the paired pleural ganglia. These small, spherical (Ø: about 300 μ) ganglia are linked to the cerebral, pedal and parietal ganglia by short connectives (length: about 150 μ). The DGC are dispersed between the ordinary neurones. Their number amounts to 10–15 in the left, and to 15–20 in the right ganglion. In addition small groups of this cell type are found in the left (2–5 neurones) and in the right (4–10 neurones) parietal ganglion (Fig. 1).

The secretory material present in the cytoplasm stains dark green with Alcian Blue/Alcian Yellow after oxydation (Pl. I fig. 1), and reacts positively to the Gomori stains for neurosecretion. The ultrastructural characteristics of these neurones have been described before (Wendelaar Bonga, 1970a). The secretory substance, consisting of elementary granules with a mean diameter of 2000 Å, is transported by the axons towards the peripheral zones of the cerebro-pleural, pleuro-parietal, and parieto-visceral connectives and to the periphery of the nuchal nerves, where they terminate adjacent to, and in, the perineurium (Fig. 1; Pl. I figs 2, 3). These areas are called neurohaemal zones, since it is here that the contents of the elementary granules are released. In a study of the daily cycle of a number of neurosecretory cell types of *Lymnaea stagnalis* it appeared that the secretory material of the DGC is released at a constant rate during the 24-hour period (Wendelaar Bonga, 1971).

Apart from the DGC, there are several hundred other neurones in the pleural ganglia. In the cytoplasm of nearly all of these neurones, granules are present. At least eight types can be distinguished. They are all smaller than the granules of the DGC, and can be easily distinguished from the latter. At least three types probably contain neurotransmitter substances, as these granules were found at neuromuscular junctions during the present study.
Fig. 1. Diagram of the central nervous system of Lymnaea stagnalis. The location of the Dark Green Cells (black dots) in the pleural (pl) and parietal (par) ganglia are indicated, as are the neurohaemal areas of these neurones at the periphery of the nuchal nerves (1) and of the cerebro-pleural (2), pleuro-parietal (3), and parieto-visceral (4) connectives (thick lines), and in the perineurium (mottled areas); cer, cerebral ganglia; visc, visceral ganglion; ped, pedal ganglia.

A. The Cell Bodies of the Dark Green Cells

For studying the rate of synthesis of the secretory material in the cell bodies of the DGC, special attention was paid to the rough endoplasmic reticulum (GER) and to the Golgi complex. Since the secretory product is proteinaceous, these organelles are likely to be involved in the synthesis of this material. The number of secretory granules was also taken into account, as were the morphology and the volume of the cytosomes and of the mitochondria.

As the cell bodies of the DGC are dispersed throughout the pleural ganglia, only part of them was found in the ultrathin sections. Consequently the sampling areas had to be small. Cross sections of about 5 neurones per animal were photographed. From the available photographs a cytoplasmic area of 2000 \( \mu^2 \) per animal was selected on the basis of technical quality of the material, and studied further. For
obtaining quantitative information concerning the GER, the mitochondria, and the cytosomes, lineal integrative analysis was applied (cf. Loud et al., 1965). A square grid of lines (distance of the lines: 15 mm) was projected onto the electron micrographs (final magnification: 18,000 x). The extent of the GER was determined by counting the number of intersections of its membranes with the sampling lines, and by substituting the figure obtained in equation 5, as given by Loud et al. (1965), which yields the length of the membranes of the GER per surface unit of cytoplasm. The total volume of the cytosomes and of the mitochondria was determined by measuring the fraction of the sampling lines projected on these organelles. According to Delesse's theorem, this fraction is equivalent to the volume fraction of these organelles in the cytoplasm (Weibel & Gomez, 1962).

Since the elementary granules of the DGC obviously arise in the Golgi zones, the extent, as well as the degree of activity, of the Golgi complex was estimated. The methods applied were similar to those introduced in a previous study (Wendelaar Bonga, 1971). As a change in the activity of the Golgi complex may be accompanied by changes of its volume (cf. Pilgrim, 1969) the number of profiles of the Golgi complex per sampling area was determined. The relative rate of the secretory activity of the Golgi complex was estimated by determining the number of "active" profiles of the complex, i.e., the number of Golgi zones in the sections showing secretory material within the saccules (Pl. II fig. 1). As the secretory material is rather electron dense it can be easily distinguished. The relation between the presence of this material in the Golgi saccules and Golgi activity has been established for many cell types (cf. Beams & Kessel, 1968).

As the elementary granules were rather scarce in the cytoplasm of most experimental groups, lineal analysis did not give satisfactory results. Therefore, the number present in the sampling area was counted.

The data was statistically tested (two-sided) for monotonic trend in location of K random samples, at the 5% probability level (de Jonge, 1963).

a. Controls

The cell bodies of the DGC were rounded or slightly oval, varying in size from 30 to 60 μ. The mean volume of their cytoplasm amounted to 2450 ± 750 μ³. The quantitative results obtained at the ultrastructural level are presented in Fig. 2 (day 0).

It appeared that the membranes of the GER showed only small cisterns and were located in small arrays. Furthermore, small numbers
of free ribosomes were found (Pl. II fig. 1). The parts of the membranes of the GER adjacent to the Golgi saccules frequently showed small evaginations. Small clear vesicles (⌀: 600–800 Å) were often observed in between the GER and the Golgi zones. Also the outer Golgi saccules often showed evaginations. These structures have been regarded as indicative of a transport system of secretory material from the GER towards the Golgi apparatus (WenDELAAR BONGA, 1971). The mean extent of the membranes of the GER, in Fig. 2A presented as the mean length of the membranes per sample area, was equivalent to about 1.2 µ² of membrane surface per µ³ of cytoplasm.

The Golgi zones showed 3–6 saccules, often slightly bent. The saccules at the outer side showed, apart from the evaginations mentioned, many fenestrations, especially at their periphery. Adjacent to the inner saccules small clear vesicles (Ⓞ: 600–1200 Å) and, occasionally, larger coated vesicles (⌀: 1000–1500 Å) occurred. Furthermore, a varying number of elementary granules was present. The mean number of Golgi zones per 2000 µ² of cytoplasm is indicated in Fig. 2B. A fair number of them showed secretory material confined within the saccules: the mean number of active Golgi zones was 48%. Acid-phosphatase activity was occasionally found in the innermost saccules and in small vesicles (⌀: 1000–1500 Å), some of which had a coated membrane. No enzymatic activity was visible in saccules containing secretory material or in elementary granules. These granules were distributed throughout the cytoplasm, slightly more concentrated around the Golgi zones than in other areas. The density of the granules was low: about 700 per 2000 µ² (Fig. 2C).

The mitochondria, evenly distributed, were usually enwrapped by membranes of the GER. Their volume comprised 6.75 ± 0.9% of the total cytoplasmic volume. As this figure was very constant in all experimental groups, it will not be mentioned further.

Three types of cytosomes were distinguished. They were regarded as different phases of the same type of organelle, since transitional stages were found. The first type, circular, oval or irregularly shaped and with a diameter between 1 and 4 µ, is characterized by an electron transparent matrix containing membranous and granular structures, identifiable as cell organelles such as membranes of the GER or mitochondria (Pl. III fig. 2). This type obviously represents digestive vacuoles. Since heterophagy is improbable in neurones (Koenig, 1969), they are regarded to be of autophagous origin. It is commonly agreed that in such vacuoles cytoplasmic areas are degraded by enzymes supplied by primary lysosomes. The latter are supposed to fuse with the vacuoles (de DUVE & WATTIAUX, 1966; ERICSSON, 1969). The second type of cytosome contains a variety of membranous and
amorphous material of different electron densities (Pl. II fig. 1). No cell organelles were observed in these cytosomes. They are regarded as being derived from the autophagous vacuoles. Apparently they represent a more progressive stage of digestion. In contrast to the autophagic vacuoles, they show a moderate to high acid-phosphatase activity and therefore, are regarded as secondary lysosomes (cf. de Duve & Wattiaux, 1966). The lamellate bodies (cf. Wendelaar Bonga, 1971) which contain densely packed and concentrically or helically arranged membranes, and which showed a moderate to high acid-phosphatase activity in their central parts, belong to this group of cytosomes. The diameter of the secondary lysosomes was 1–3 \( \mu \) although it occasionally exceeded 6 \( \mu \). This type is comparable to the phospholipid and mixed lipid droplets described by Meeke & Lane (1964) in neurones of Helix aspersa. The third type of cytosome in the DGC is relatively small (\( \phi : 0.3–1.5 \mu \)) and has a homogeneous, rather electron dense content (Pl. II fig. 2). No acid-phosphatase activity is observed in the organelles of this type. They are regarded as residual bodies, the final stage of the lysosome cycle (de Duve & Wattiaux, 1966).

In the controls the autophagous vacuoles were almost completely absent. Lineal analysis revealed that the volume of the three types of cytosomes was on an average 0.5% of that of the cytoplasm. In the controls most of them were residual bodies, which made up about 60% of the total cytosomal volume (Fig. 2D).

b. Snails exposed to de-ionized water (Fig. 2)

The osmolalities of the blood and of the media, as well as the body weight of snails exposed to de-ionized water are presented in Table I. The osmolality of the blood rapidly decreased in the first 24 hours of exposure, but subsequently increased gradually during the experiment. The mean body weight also showed an initial decrease, which was probably due to a diminished water content of the snails, as they looked rather shrunken at day 1. At day 8 and 15, when the body weight was only slightly below that observed at day 0, the snails had regained their normal appearance. Oviposition did not occur during the first four days, but was resumed thereafter, although the total number of egg masses produced per animal was half that of the controls. The amount of lettuce consumed was about 25% above control level.

Day 1 (24 hours in de-ionized water).—The morphology of the GER only showed minor changes. The mean length of the membranes per surface unit was slightly higher when compared to that of the controls (Fig.
Fig. 2. Quantitative data concerning the cell bodies of the DGC of 5 groups of 5 animals each; 3 groups were fixed after exposure to de-ionized water for 1, 8, or 15 days, respectively; one group was fixed at day 22, after exposure to de-ionized water for 15 days and, afterwards, to tap water for a week; a control group, kept in tap water, was fixed at day 0 (squares). Per animal a cytoplasmic area of 2000 µ² was sampled. A: length of the membranes of the granular endoplasmic reticulum (GER); B: total number of Golgi zones (black symbols) and of the number of “active” Golgi zones (open symbols); C: number of elementary granules; D: percentual volumes of the total number of cytosomes (black symbols) and of the residual bodies (open symbols). The mean values per group (± S.E.) are indicated.
TABLE I
Changes of the body weight and the osmolalities of the blood of groups of 10 snails, and the osmolalities of the media to which the snails had been exposed. The blood was analyzed of a group kept in tap water (controls, day 0), of 3 groups exposed to de-ionized water for 1, 8 or 15 days, and of a group exposed to de-ionized water for 15 days and, afterwards, to tap water for a week (day 22).
The percentual changes of the weight of the soft parts of the body of a group of snails, compared to the weights before the experiment, were determined during exposure to de-ionized water for 15 days (at day 1, 8 and 15) and afterwards, to tap water for a week (at day 22).
The mean values per group (± S.E.) are presented.

<table>
<thead>
<tr>
<th></th>
<th>Change in body weight (%)</th>
<th>Osmolality of the blood (mOsm/kg H₂O)</th>
<th>Osmolality of the medium (mOsm/kg H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (day 0)</td>
<td></td>
<td>116.2 ± 2.2</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>de-ionized water (day 1)</td>
<td>-5.3 ± 2.8</td>
<td>86.4 ± 6.0</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>de-ionized water (day 8)</td>
<td>-1.9 ± 1.8</td>
<td>94.2 ± 4.2</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>de-ionized water (day 15)</td>
<td>-1.2 ± 1.3</td>
<td>110.6 ± 3.7</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>de-ionized water/tap water</td>
<td>-2.0 ± 1.6</td>
<td>118.4 ± 6.2</td>
<td>16.2 ± 0.2</td>
</tr>
</tbody>
</table>

2A). The total extent of the Golgi complex had not changed. On the other hand, there was more secretory material present within the Golgi saccules: the mean number of active Golgi zones had increased from 48% in the controls to 62% (Fig. 2B). The mean number of elementary granules showed a fall of about 30%. This fall, at a moment when the Golgi activity had apparently increased, indicates that during the first 24 hours the rate of transport of the granules towards the axons had grown, in a way surpassing the rate of increase of their formation. The granules were primarily found around the Golgi zones. The total volume of the cytosomes had not changed. As in the controls, the majority consisted of residual bodies. Autophagous vacuoles were not observed.

Day 8.—The GER was markedly enlarged, viz., by 70% when compared to the controls (Pl. II fig. 2). The activity of the Golgi complex was at a level slightly higher than that noted at day 1, whereas its size was still unaltered. The number of elementary granules in the cytoplasm had increased when compared to day 1. The fractional volume of the cytosomes was slightly higher than that of the controls.
Day 15.—A further increase of the extent of the GER, nearly doubled when compared to the controls, was evident. The Golgi complex was still unchanged in size. A further increase of the number of active Golgi zones, to about 70%, was found. The number of elementary granules in the cytoplasm was still growing. The amount and the distribution of acid-phosphatase activity in the Golgi complex and in the cytosomes was similar to those in the controls. The fractional volume of the cytosomes had increased further. With the light microscope no changes were observed in the staining intensity of the cytoplasm with Alcian Blue/Alcian Yellow. The cell bodies had grown considerably, however. The mean volume of the cytoplasm per cell body had increased by about 80%, to 4500 ± 1750 µ³.

Day 22 (one week after replacement of the snails in tap water).—Most of the effects caused by the exposure to de-ionized water had disappeared. The extent of the GER, as well as the number of active Golgi zones was reduced to a level slightly below that of the controls. Conversely, an increase of the fractional volume of the cytosomes, by more than 200% when compared to the volume observed at day 15, was noticed. Further analysis made clear that this rise was primarily due to a volume increase of the autophagic vacuoles and of the secondary lysosomes. The volume of the residual bodies nearly doubled.

Light microscopic measurements showed that the mean volume of the cytoplasm per cell body had returned to control values (3100 ± 1050 µ³).

It is apparent that the quantitative analysis revealed notable changes in the cytoplasm of the DGC during exposure to de-ionized water. The extent of the GER, as well as the number of active Golgi zones per surface area increased considerably during the first 15 days. These trends were statistically significant (p < 0.001 and p < 0.01, respectively). The number of elementary granules in the cytoplasm also increased significantly (p < 0.01) during this experiment. The changes in the fractional volume of the cytosomes and of the mitochondria were statistically not significant.

For evaluating these results, the enlargement of the volume of the cytoplasm of the cell bodies (by 80%) has to be taken into account. The described changes then become even more prominent: per cell body there was an almost fourfold increase of the extent of the GER and of the number of active Golgi zones, a threefold increase of the number of elementary granules, and an increase by 80% of the extent of the Golgi complex and of the volumes of the cytosomes and of the mitochondria. Exposure to de-ionized water leads obviously to hyperactivity of the DGC.
The threefold increase of the cytosomes, observed at day 22, is only partly due to the reduction of the volume of the cytoplasm. Probably, this decrease is caused by cellular autophagy, as the number of autophagic vacuoles was much higher than observed before.

c. Snails exposed to 0.1 M sodium chloride (Fig. 3)

The osmolalities of the blood and of the media, as well as the percentual changes of the body weight of the snails exposed to saline are presented in Table II. The osmolality of the blood increased rapidly during the first hours of exposure to this highly hyperosmotic medium. It remained constant and slightly hypertonic to the solution during the experiment. The body weight was initially enhanced by almost 5%, probably due to a net water uptake. The snails had a swollen appearance at day 1. The swelling disappeared within some days. As in the first experiment, oviposition stopped during the first days, and was resumed later at a decreased level. Some of the animals had their penis constantly extruded. The food consumption was about 30% above

<table>
<thead>
<tr>
<th></th>
<th>Change in body weight (%)</th>
<th>Osmolality of the blood (mOsm/kg H$_2$O)</th>
<th>Osmolality of the medium (mOsm/kg H$_2$O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (day 0)</td>
<td></td>
<td>116.2 ± 2.2</td>
<td>15.8 ± 0.4</td>
</tr>
<tr>
<td>0.1 M NaCl (day 1)</td>
<td>4.6 ± 3.3</td>
<td>189.6 ± 2.8</td>
<td>188.8 ± 0.2</td>
</tr>
<tr>
<td>0.1 M NaCl (day 8)</td>
<td>1.7 ± 2.3</td>
<td>191.6 ± 3.0</td>
<td>189.0 ± 0.3</td>
</tr>
<tr>
<td>0.1 M NaCl (day 15)</td>
<td>2.1 ± 2.0</td>
<td>192.2 ± 1.4</td>
<td>188.4 ± 0.2</td>
</tr>
<tr>
<td>0.1 M NaCl/tap water (day 22)</td>
<td>-1.1 ± 1.2</td>
<td>118.2 ± 4.1</td>
<td>16.0 ± 0.3</td>
</tr>
</tbody>
</table>
Fig. 3. Quantitative data concerning the cell bodies of the DGC of 5 groups of 5 animals each; 3 groups were fixed after exposure to a 0.1 M sodium chloride solution for 1, 8, or 15 days, respectively; one group was fixed at day 22, after exposure to the sodium chloride solution for 15 days and, afterwards, to tap water for a week; a control group, kept in tap water, was fixed at day 0 (squares). Per animal a cytoplasmic area of 2000 μ² was sampled. A: length of the membranes of the granular endoplasmic reticulum (GER); B: total number of Golgi zones (black symbols) and the number of “active” Golgi zones (open symbols); C: number of elementary granules; D: percentual volumes of the total number of cytosomes (black symbols) and of the residual bodies (open symbols). The mean values per group (± S.E.) are indicated.
that of the controls. The quantitative results of this experiment are presented in Fig. 3.

Day 1 (24 hours in 0.1 M sodium chloride).—Changes were already notable after 24 hours. The extent of the GER and of the Golgi complex had not markedly changed, but the Golgi saccules contained a diminished amount of secretory material. The number of active Golgi zones decreased from 48%, in the controls, to 17% (Fig. 3B). Thus, a decreased rate of granule formation was indicated. There was an accumulation of elementary granules in the cytoplasm. The mean number per surface unit had almost doubled during the first 24 hour period (Fig. 3C). Thus, less secretory material was transported by the axons than was produced by the Golgi zones. The fractional volume of the cytosomes had not changed (Fig. 3D).

Day 8.—Marked alterations were found. The extent of the GER was reduced by about one third. The membranes of the GER were less densely packed and also less densely studded with ribosomes than those in the controls, whereas the number of free ribosomes had increased considerably (Pl. III fig. 1). Apparently many bound ribosomes had become free from the membranes. The size of the Golgi complex, as well as the amount of secretory material in its saccules was similar to that observed at day 1. The number of elementary granules showed a distinct decrease. Striking changes were observed in the number and the morphology of the cytosomes. A sevenfold increase of their fractional volume appears from Fig. 3D. Autophagous vacuoles, almost absent before, were generally found (Pl. III fig. 2), as were younger stages of the secondary lysosomes. In contrast, the number and volume of the residual bodies remained constant.

Day 15.—After two weeks of exposure to saline the degenerative tendencies became even more apparent. The extent of the GER was further reduced, to about half that of the controls. The membranes occurred in loosely arranged stacks, or singly, around the mitochondria. Large parts of the cytoplasm were devoid of cellular organelles. The number of elementary granules was still lower than at day 8. Occasionally structures reminiscent of elementary granules were observed within cytosomes. This may indicate that lysosomal digestion of secretory material (granulolysis, cf. Farquhar, 1969) did occur in the DGC. The total number of Golgi zones, as well as the number of active Golgi zones had not changed further. Stages of formation of elementary granules, although hard to find, were not completely absent. The acid-phosphatase activity in the Golgi zones was high
when compared to the controls, where it was found to be rather low or absent. Frequently large lead deposits were observed, especially in the innermost one or two saccules of the Golgi zones (Pl. IV fig. 2) and occasionally in small vesicles, which may represent primary lysosomes. The number of elementary granules was slightly lower than observed at day 8.

Extremely high numbers of cytosomes were found, particularly secondary lysosomes (Pl. IV fig. 1). Furthermore, a low number of autophagous vacuoles was observed. Acid-phosphatase activity was moderate to high in the former (Pl. IV fig. 3), low or absent in the latter type of cytosome. The absence of enzyme activity in autophagous vacuoles may indicate that these cytosomes were newly formed and had not yet fused with primary lysosomes. The mean volume of the residual bodies was enhanced when compared to that of the controls.

In a number of mitochondria the cristae had partly or completely (Pl. IV fig. 1) disappeared. No changes were visible in the matrix of these mitochondria. These alterations may represent the first stages of mitochondrial degeneration. In involuting kidney cells of *Lymnaea stagnalis* (Wendelaar Bonga & Boer, 1969) a similar process has been described which led to the formation of lipid globules. This transformation has also been reported for a number of other animals (cf. Ratcliffe & King, 1969), but was not observed in the DGC.

The DGC could not be detected with certainty with the light microscope, since their cytoplasm was hardly stained with the Alcian Blue/Alcian Yellow technique. This is a further indication that the amount of secretory material present in the GER, the Golgi zones and in the elementary granules had drastically diminished. As a consequence, no light microscopical data about the cell size could be obtained from this experimental group. Therefore, electron micrographs were used for measurements on these neurones. Although it was not possible to determine the size of the cell bodies very accurately at the ultrastructural level, the results suggested a substantial decrease of the mean volume of the cell bodies, by at least 50% when compared to similar data concerning controls.

Day 22.—One week after replacement of the snails in tap water the morphological and quantitative characteristics of the cell bodies of the DGC indicated a marked recovery. In Alcian Blue/Alcian Yellow stained paraffin sections the cell bodies were prominent by the deep green colour of their cytoplasm. The mean volume of the cytoplasm per cell body had returned to normal values (2800 ± 750 μ³). The extent of the GER had increased to just above control level, as had the number of active Golgi zones. An increase of the number of elementary granules
Fig. 1. Control. Light micrograph of a right pleural ganglion, showing three cell bodies of Dark Green Cells (DGC), and many non-neurosecretory neurones; np, neuropile; cs, cephalic sinus (Alcian Blue/Alcian Yellow).

Fig. 2. Control. Light micrograph of the wall of the anterior aorta (a), near the central nervous system. The muscular wall surrounding the elastic layer (el) is invaded by axonal terminations containing secretory material of the DGC (arrows) and of another type of secretory neurones, the Light Yellow Cells (LYC); GC, connective tissue cells (Granular Cells); cs, cephalic sinus (Alcian Blue/Alcian Yellow).

Fig. 3. Control. Light micrograph of a longitudinal section of a right pleuro-parietal connective. In the periphery of the connective (c) and in the perineurium (pe) axon terminals containing secretory material of the DGC are present (arrows); cs, cephalic sinus (Alcian Blue/Alcian Yellow).
Fig. 1. Control. Electron micrograph of the cytoplasm of a DGC. The membranes of the granular endoplasmic reticulum (GER) occur isolated or packed in small arrays; c, cytosome (secondary lysosome type); eg, elementary granule. Inset: "active" Golgi zone; electron dense accumulations of secretory material (arrows) are present within the Golgi saccules; cv, clear vesicles.

Fig. 2. De-ionized water, day 8. Densely packed membranes of the granular endoplasmic reticulum (GER) dominate in the cytoplasm of a cell body of the DGC. The membranes are heavily studded with ribosomes; c, cytosome (residual body); eg, elementary granules.
Fig. 1. 0.1 M Sodium chloride solution, day 8. Membranes of the granular endoplasmic reticulum (GER) occur isolated in the cytoplasm of a cell body of the DGC. They are sparsely studded with ribosomes. Large numbers of free ribosomes are present; c, cytosomes (secondary lysosomes); GZ, "inactive" Golgi zone: no secretory material is present within the Golgi saccules.

Fig. 2. 0.1 M Sodium chloride solution, day 8. Part of the cytoplasm of the cell body of a DGC. Several autophagic vacuoles (av), showing lamellar and vesicular structures and ribosomes, are present, as are secondary lysosomes (sl) and many free ribosomes; eg, elementary granule.
Fig. 1. 0.1 M Sodium chloride solution, day 15. The cytoplasm of a cell body of the DGC, showing cytosomes (c) of the secondary lysosome type. The mitochondria (mi) are devoid of cristae. Many free ribosomes are present.

Fig. 2. 0.1 M Sodium chloride solution, day 15. Acid-phosphatase test. Large lead deposits (arrows) reveal the presence of acid-phosphatase activity in the two inner saccules of a Golgi apparatus in a cell body of the DGC.

Fig. 3. 0.1 M Sodium chloride solution, day 15. Acid-phosphatase test. The finely granular lead deposits (arrows) indicate the presence of acid-phosphatase activity in two secondary lysosomes (sl) in a cell body of the DGC; eg, elementary granules.
Fig. 1. Control Axon terminals containing elementary granules of the DGC (DGC) and of the Yellow Cells (YC), as well as axon profiles devoid of granules (ax) are present in the periphery of a cross section of the right pleuro-parietal connective; gl, extension of a glial cell; c, glial cytosome; pe, perineurium.

Fig. 2. De-ionized water, day 1. Axon terminals containing elementary granules of the DGC are present in the periphery of the right pleuro-parietal connective. An omega-shaped membrane indentation (arrow) indicates release of the secretory material of a granule; tvs, tubular and vesicular structures; pe, perineurium.

Fig. 3. De-ionized water, day 1. Axon terminals containing elementary granules of the DGC, at the periphery of the right pleuro-parietal connective. The presence of an electron dense granule core in the interaxonal space (arrow) indicates release by exocytosis; tvs, tubular and vesicular structures; pe, perineurium.
Fig. 1. 0.1 M Sodium chloride solution, day 1. An axon terminal (ax) crowded with elementary granules of the DGC is present in the perineurium around the right pleuro-parietal connective; bs, blood space; m, muscle fibres.

Fig. 2. 0.1 M Sodium chloride solution, day 15. Axon terminals (ax) presumably of the DGC, which are devoid of elementary granules, are present in the periphery of the right pleuro-parietal connective; c, cytosomes (residual bodies); pe, perineurium.

Fig. 3. 0.1 M Sodium chloride solution, day 8. Axon terminal of the DGC, showing elementary granules (eg), multilamellate cytosomes (c), and concentrically arranged tubular lamellae; mi, mitochondrion. Inset: autophagous vacuole, containing elementary granules in an axon terminal of the DGC.
was also noticed. Autophagous vacuoles had disappeared. Most cytosomes were of the lamellate and secondary lysosome type. No degenerative stages of the mitochondria were found.

The most obvious changes induced in the DGC of the snails exposed to hyperosmotic saline may be summarized as a reduction of the GER and of the number of active Golgi zones, per surface unit. Both trends were statistically significant (p < 0.01 and p < 0.001, respectively). There is a change in the function of the Golgi complex from the formation of elementary granules to the production of lysosomal enzymes. A significant decrease of the number of elementary granules per surface unit (p < 0.01) and an highly significant rise of the fractional volume of the cytosomes (p < 0.0001) occurred during the experiment. These changes, together with the reduction of the mean volume of the cytoplasm of the cell bodies to at least half the control value, are highly indicative of a reduction of the secretory activity. The occasional occurrence of some neurosecretory material within the Golgi lamellae may, however, mean that the formation of elementary granules has not been suppressed completely.

The changes observed at day 22 reveal that the effects induced by saline are reversible. The decrease of the fractional volume of the cytosomes, when compared to day 15, although partly due to the increase of the cytoplasmic volume, indicates that much of the cytoplasmic material confined within these bodies at day 15 had been digested. The duplication of the volume of the residual bodies, when compared to the controls, confirms this conclusion.

B. The Axons of the Dark Green Cells

The neurosecretory material of the DGC was quantitatively studied in that part of the neurohaemal area of these neurones where the axon terminals are most numerous, i.e., in the periphery of the connective between the right pleural and parietal ganglia. In previous investigations (Wendelaar Bonga, 1970a, 1971) the morphological aspects of storage and release of the elementary granules of the DGC, among other cell types, have been studied. Evidence was presented that the release of the secretory substance from the axons into the bloodstream takes place by exocytosis, in those parts of the axon terminals which face the perineurium. This was concluded from the presence of omega-shaped membrane indentations, some of which still enclosed the contents of the granules (cf. Wendelaar Bonga, 1970a, Fig. 26). Apart from these indentations the presence of small clear vesicles in the axon terminals was regarded as indicative for release of neurosecretory material.
The amount of secretory material stored in the periphery of the right pleuro-parietal connective was estimated by counting the number of axon profiles containing elementary granules of the DGC type. The number of these axon profiles in three cross sections of the connective, cut at distances of about 50 \( \mu \) from each other, were added. A number of these axons showed release phenomena. This number, determined in the same sections, was used as a measure for determining the rate of release of the secretory material. The data is presented in Fig. 4.

![Graph showing quantitative data](image)

Fig. 4, A and B. Quantitative data concerning the neurohaemal zones of the DGC of 9 groups of 5 animals each; 3 groups were exposed to de-ionized water (A) and 3 other groups to a 0.1 M sodium chloride solution (B), for 1, 8, and 15 days, respectively; 2 groups were fixed at day 22, after exposure to de-ionized water (A) or the sodium chloride solution (B) for 15 days and, afterwards, to tap water for a week; a control group, kept in tap water, was fixed at day 0. Per animal 3 cross sections of the right pleuro-parietal connective were sampled. The total number of peripherally located axon profiles containing elementary granules of the DGC (black symbols) and the number of those axons showing release phenomena (open symbols) are indicated (mean values per group, ± S.E.).

a. Controls

At the surface of the connective four types of granule containing axon terminals occur. They are irregularly distributed. Part of them face the perineurium, while another part is separated from the perineurium
by other axons or processes of filamentous glial cells. Due to their morphological characteristics the terminals of the DGC are easily distinguished from the other types of axon endings (cf. WENDELAAR BONGA, 1970a). The axon endings of the DGC were usually completely filled with elementary granules (Pl. V fig. 1). About 27% of the axon endings showed release phenomena (Fig. 4, day 0).

b. Snails exposed to de-ionized water (Fig. 4A)

Day 1 (24 hours in de-ionized water).—The number of granule-filled axon profiles was reduced by about 35%. Moreover, the axons were less densely packed with granules than those of the controls. Apparently the rate of release of the secretory material was enhanced during the first 24 hours. The number of axon profiles showing release phenomena has slightly increased, and amounted to 50% of the granule containing axons. In a fair number of them tubular and vesicular structures were observed (Pl. V figs 2, 3). In a previous study (WENDELAAR BONGA, 1971) these structures were found in axons which were in a late stage of the release process. They were seldom found in control animals.

Day 8 and 15.—On these days a gradual increase of the number of granule containing axons and of the number of these axons showing release phenomena was noted. Also in these releasing axons many tubular and vesicular structures were found.

Day 22 (one week after replacement in tap water).—The number of granule-filled axons had returned to that of the controls. The mean number of releasing axons was reduced to below control level, from 60%, at day 15, to 16%.

It may be concluded from the results that already at the end of the first 24 hour period of exposure to de-ionized water a new relationship between accumulation and release of the secretory material was established. This relationship was only slightly changed during the further course of the experiment and is indicative of a higher release activity. The tendency for the release activity to increase was statistically significant (p < 0.05). The variation in the number of granule containing axons did not, however, show a statistically significant trend. The results obtained from the study of the axon terminals of the DGC are in line with the results of the cell bodies: in both parts of the neurones an increased activity was found.
c. Snails exposed to 0.1 M sodium chloride (Fig. 4B)

Day 1 (24 hours in 0.1 M sodium chloride).—As in the former experiment, striking changes were already observed after 24 hours of exposure. A marked accumulation of secretory granules was observed. The mean number of granule containing axons had increased by 80% when compared to the controls. Furthermore, most of them were crowded to a very high degree, usually not observed in control animals. Such axons were also observed in the perineurium (Pl. VI fig. 1). This accumulation was obviously related to a reduction in the rate of release of the secretory material, as was concluded from the reduction of the percentage of terminals showing release phenomena, from 27% in the controls, to about 8% in the experimental snails.

Day 8.—The amount of secretory material stored in the axon terminals was reduced when compared to day 1. As the rate of release was obviously very low, this reduction will only partly have been due to exocytosis of the elementary granules. Probably a further reduction was caused by intra-axonal digestion of elementary granules. This was indicated by the presence of two types of structures which were only rarely found in controls. The first structure consisted of clusters of elementary granules enclosed by one or two membranes (Pl. VI fig. 3). It may represent an autophagous vacuole. The other type consisted of numerous concentrically arranged membranes enwrapping elementary granules (Pl. VI fig. 3). These latter structures were rather similar to the multilamellate organelles described before in axons of neurosecretory cells (cf. Knowles, 1964; Wendelaar Bonga, 1971). The presence of multilamellate organelles in this stage of the experiment suggests that they are involved in granule degradation.

Day 15.—After two weeks of exposure to saline the stores of elementary granules had been further reduced. Secondary lysosomes and residual bodies were found in fair numbers, particularly in terminals almost devoid of elementary granules (Pl. VI fig. 2). Since at day 8 autophagic vacuoles were observed which were involved in the breakdown of elementary granules, the cytosomes present at day 15 were interpreted as further stages in the process of granule degradation. The other types of granule containing axon terminals did not show any notable morphological alteration during the experiment.

Day 22 (One week after the replacement in tap water).—The morphology of the DGC terminals was similar to that of the control animals. The mean number of granule containing axons was slightly below control
level. The number of axons showing release phenomena was just above that of the controls.

It was concluded that the release activity was strongly reduced during the experiment in saline. This trend was statistically significant (p < 0.05). Although there was an accumulation of elementary granules at day 1, later a considerable decrease occurred, which was statistically significant (p < 0.05).

2. OTHER TYPES OF NEUROSECRETORY CELLS

In all the experimental groups the degree of tissue preservation was similar. Thus, it may be concluded that the changes in the DGC were not due to a general tissue damage caused by the experimental treatment. To establish whether these changes were specific responses of the DGC, two other types of neurosecretory cells were taken into consideration. Groups of the Light Yellow Cells and of the Yellow Green Cells (cf. Wendelaar Bonga, 1970a), located in the right parietal ganglion, were studied in 3 control animals and in 4 groups of 3 animals fixed after 1 or 15 days of exposure to de-ionized water or saline, respectively. There were neither alterations in the number of elementary granules in the cell bodies of these neurones, nor in the quantitative aspects of the GER, the Golgi complex or the cytosomes. This conclusion was based on the results of quantitative analysis of small cytoplasmic areas (about 1000 μ² per animal).

IV. DISCUSSION

1. METHODS

In the present study the integrative lineal analysis method (cf. Weibel & Gomez, 1962; Loud et al., 1965) was used for the determination of the extent of the GER and of the volumes of the cytosomes and of the mitochondria. Due to technical limitations and because only a small number of neurones could be studied in each animal, the size of the sampling areas was limited. As a consequence the method was not adequate for measuring the volume of the elementary granules, since their number was too low. However, the size of these granules is fairly uniform (Wendelaar Bonga, 1970a). Therefore, the information obtained in this study by determining the number of granules per surface unit is comparable to that yielded by lineal analysis. Since the measurements regarding the GER are based on the number of intersections of the membranes with the sampling lines, the membranes
orientated in the plane of sectioning have not been taken into account. The resulting underestimation of the membrane surface will, however, be similar for all experimental groups.

The methods used for quantifying the Golgi complex and its activity have been discussed before (Wendelaar Bonga, 1971).

The spread of the data concerning the cell bodies was rather small, especially when the limited size of the sampling areas is taken into account. This spread was rather similar in all groups of snails, which indicates that the DGC are functionally synchronized to a high degree, in the experimental groups as well as in the controls. It is well known that functional synchronization may be induced by experimental treatment, but in normal conditions the rate of secretory activity between cells of the same type often varies considerably, even within the same animal (Liotti & Rosi, 1968; Picard, 1970).

2. THE CELL BODIES OF THE DARK GREEN CELLS

Since in invertebrates only light microscope studies on osmotically induced changes in neurosecretory cells have been performed so far, the present results will primarily be compared with ultrastructural studies dealing with the vertebrate hypothalamo-neurohypophyseal system. The neurones in this system are involved in osmotic regulation: they respond with increased hormone synthesis to a rise in the blood osmotic pressure. The coinciding changes in the cytoplasm have been extensively studied with the electron microscope, especially in the rat (Nemetschek-Gansler, 1965; Streefkerk, 1967; Rechardt, 1969). In this animal also lineal analysis has been applied at the ultrastructural level (Reinhardt et al., 1969a, b). These neurones are inactivated by overhydration, as was studied with the light microscope (cf. Vilhardt, 1970).

On the basis of the quantitative results regarding the synthesis and release of the secretory material, it was concluded that exposure of *Lymnaea stagnalis* to de-ionized water activates the DGC, whereas a stay in saline leads to hypoactivity. From the observed changes in the volume of the cytoplasm of these neurones after two weeks of experimental treatment—a duplication of the volume in de-ionized water, a reduction to at least half the control value in saline—it can be concluded that there is a positive relationship between the secretory activity of the cell and its volume. This inference is in accordance with the results obtained for many glandular cells, including vertebrate secretory neurones (Streefkerk, 1967; Reinhardt et al., 1969a, b; Oliverneau, 1970).

Exposure to de-ionized water results in a duplication of the relative
extent of the GER of the DGC. No striking changes were observed in the width of the cisterns. On the other hand activation of the hypothalamic neurosecretory nuclei resulted in an increase of the width of the cisterns of the GER (Nemetschek-Gansler, 1965; Streefkerk, 1967). Furthermore, in the rat the fractional volume of the GER did not vary, at least during the first five days, as was determined by Reinhardt et al. (1969a). These latter authors concluded that there is a fixed relationship between the extent of the GER and the cytoplasmic volume. Such a relationship is obviously absent in the DGC.

The rise in the number of free ribosomes after inactivation of the DGC in saline is assumed to be due to the detachment of ribosomes from the membranes of the GER. In inactivated protein-producing glandular cells this phenomenon is well known, e.g., in the neurosecretory Q-cells of the oligochaete Enchytraeus albidus (Gersch & Ude, 1970). It is regarded as a degenerative feature. It is understandable that this process is observed after reduction of the rate of formation of the secretory granules, since there is evidence that bound ribosomes in glandular cells are highly specialized for the production of the secretory product (cf. Gaye & Denamur, 1970).

The number of Golgi zones per surface unit, as well as the fractional volume of the mitochondria, were similar in all experimental groups. This means that the changes occurring per cell body in the extent of the Golgi complex and in the volume of the mitochondria were proportional to the volume changes of the cytoplasm. The absence of a reduction of the Golgi complex of the DGC after inactivation in saline may be related to the observation that in this medium the synthetic activity of the Golgi zones was not suppressed. A shift was noted from the formation of secretory granules towards the elaboration of primary lysosomes. Already one day after the start of the experiments the percentage of secretory active Golgi zones had markedly changed. At that time it reached a level which remained constant in saline and which showed only minor alterations in de-ionized water during the remaining experimental period. In short-term experiments the changes in Golgi activity are obviously a more sensitive indicator of changed secretory activity than the alterations in the GER. The extent of the membranes as well as the morphology of the GER had hardly changed after the first 24 hour period. The same inference was drawn in a study of the daily secretory rhythm of the Caudo-Dorsal Cells of Lymnaea stagnalis (Wendelaar Bonga, 1971).

In the experiments the changes in the number of secretory granules in the cell bodies and in their axon terminals ran parallel. The most striking changes were observed at day 1. The fall of the number of granules in activated neurones, as well as the rise observed after inac-
tivation are consistent with the experimentally induced variations in the amount of secretory material described in some other neuroendocrine cell types. As in the DGC, a decrease of the amount of secretory material in the cell bodies (Gersch & Ude, 1970; Reichardt, 1969; Vilhardt, 1970) and in the axon terminals (Reinhardt et al., 1969b; Vilhardt, 1970) is accompanying an increase of the secretory activity, whereas an experimentally induced accumulation of elementary granules runs parallel with a decrease of the secretory activity (Vilhardt, 1970). These observations show that during the first stages of the processes of activation and inactivation the rates of transport and release of elementary granules are influenced to a higher degree than the rate of production. At days 8 and 15 the relationship between the rates of production, transport and release of the elementary granules had obviously changed, as the amounts of secretory material had again increased in the activated neurones. The fall of the number of elementary granules, following the initial increase, in neurones of snails in saline is obviously partly due to autophagous digestion. Thus, the degree of accumulation of secretory material in cells which differ in their secretory activity is affected by different cellular processes. In fact, activated as well as inactivated cells may be crowded with, or be almost devoid of elementary granules. Therefore, no definite conclusions regarding the secretory activity can be drawn on the basis of the amount of secretory material present in the cells (cf. Highnam, 1965; Mordue, 1967; Wendelaar Bonga, 1971).

In the present study the cytosomes were regarded as autophagous lysosomes. In molluscan neurones these bodies, known as pigment droplets, globules or granules (cf. Chou, 1957; Boer, 1965), have been extensively investigated. In a study of the pulmonate snail *Helix aspersa* Meek & Lane (1964) identified these bodies at the ultrastructural level, and referred to them as lysosomes, since they showed acid-phosphatase activity. Nolte et al. (1965), who investigated the prosobranch snail *Crepidula fornicata*, denied that the cytosomes are concerned with the degradation of cytoplasmic material. They regarded them as stores for metabolically significant substances. An oxidative function, similar to that of mitochondria was proposed by Zs-Nagy & Kerpel-Fronius (1970) in a study of the neurones of the bivalve mollusc *Anodonta cygnea*. As in mitochondria they demonstrated succinic dehydrogenase activity in some cytosomes. The observed enzymatic activity in the cytosomes may, however, be of mitochondrial origin and point to degradation of these organelles inside lysosomes. Benjamin & Peat (1969) attributed a secretory function to the cytosomes of the pulmonate *Planorbarius corneus*, and assumed that they correspond to the Gomori-positive secretory substance of neurosecretory cells. They
ignored, however, the histochemical evidence presented by Boer (1965) for *Lymnaea stagnalis* that the composition of the secretory material is different from that of cytosomes.

The present morphological and histochemical results on *Lymnaea* confirm the supposition of Meek & Lane (1964) that cytosomes represent lysosomes. Köning (1969) concluded that they had an autophagous origin in vertebrate neurones, as has been suggested for gastropods by Chalazonitis (1968), who studied their formation in *Helix pomatia*. Their origin and development fits in with the scheme given by de Duve & Wattiaux (1966) for autophagous digestion. The absence of acid-phosphatase activity from autophagous vacuoles which contain rather intact cytoplasmic material, is in line with their view that the hydrolytic enzymes are incorporated in the autophagous vacuoles by fusion with primary lysosomes formed in the Golgi zone. Accordingly, the qualification of acid-phosphatase containing cytosomes as secondary lysosomes seems justified. Autophagy is regarded as a cellular mechanism for the breakdown of worn-out or superfluous organelles. It occurs normally in metabolically active cells or after experimentally induced reduction of the cellular activity, as an adjustment to a new functional situation (Ericsson, 1969). The small increase of the cytosomal volume in activated DGC, as observed in animals exposed to de-ionized water, may indicate an enhanced turnover of organelles. On the other hand, the more prominent increase after return to tap water may reflect a partial degradation of the cytoplasm, which can be considered as an adaptation to lowered secretory demands. Obviously the same process accounts for the five-fold volume growth of the cytosomes per cell body as observed after saline treatment. The decrease of their number and volume and the rise of the number of residual bodies, after replacement of the animals to tap water, can be interpreted as the final stage of lysosomal development as described by Ericsson: after degradation of the enclosed material the secondary lysosomes merge, and eventually only a relatively small number of residual bodies containing non-digestible residues is left.

3. THE AXONS OF THE DARK GREEN CELLS

The morphological aspects of release of elementary granules and the method to quantify this process, have been discussed before (Wende laar Bonga, 1970a; 1971). After two weeks of exposure to de-ionized water the release activity had increased with about 50%. This increase is rather low when compared to the marked growth, to fourfold, of the GER. A much higher increase of the release was
expected. Probably this discrepancy is caused by the method used to determine the release activity. As the number of axons showing release phenomena was taken as a parameter, differences in the release intensity between the releasing axons were not considered. This procedure probably leads to an underestimation of the total release, as there were more release phenomena per axon in snails exposed to de-ionized water than in control animals.

In the controls cytosomes appeared to be almost absent from the axon terminals. Contrarily, they occur normally in large accumulations, visible as Herring bodies in the light microscope in the rat neurosecretory axons (Pilgrim, 1970). Recently they have been described in the terminals of these axons by Whitaker et al. (1970). These authors suggested that these cytosomes are involved in digestion of remnants of the granule membranes which remain after exocytosis. It has been doubted whether in Lymnaea stagnalis lysosomal enzymes function in this way in the final stage of the release process (Wendelaar Bonga, 1971). As the cytosomes become more numerous in the terminals after saline exposure, it is suggested that they act in Lymnaea stagnalis primarily in removing superfluous elementary granules.

4. FUNCTIONAL SIGNIFICANCE OF THE DARK GREEN CELLS

In de-ionized water the snails appeared to maintain a high blood osmotic value, which gradually increased during the experiment and was just below the control level at day 15. As exposure of fresh water animals is known to lead to an osmotic water uptake, a swelling of the snails was expected. However, a shrinkage of the snails, and a decrease of their body weight was observed, which indicated a loss of water. A similar observation was reported by Greenaway (1970). As passive water loss is improbable in de-ionized water, a strongly increased urine production will account for the loss of water. An enforced diuresis was expected on the basis of the investigations of Van Aardt (1968). In the 0.1 M sodium chloride solution the blood was almost isotonic with the medium. Therefore, as the rate of urine formation is positively correlated with the height of the osmotic gradient between blood and medium (van Aardt, 1968) the rate of diuresis will have been drastically reduced in this solution.

The responses of the DGC in de-ionized water and in saline-hyperactivity in the first, hypoactivity in the latter solution are consistent with the hypothesis that these neurones are engaged in the control of diuresis. The substance produced in these cells may be identical to the diuretic factor of the pleural ganglia, which was demonstrated by Hekstra & Lever (1960) and Lever et al. (1961). However, more
experimental information is needed to clarify the function of these neurones. It is possible that they accomplish an increased rate of urine formation by the secretion of a cardio-accelerator substance. Since the primary urine of *Lymnaea stagnalis* is, as in other gastropods, probably produced in the pericardial cavity by ultrafiltration of the blood across the heart wall (cf. van Aardt, 1968), acceleration of the rate of the heart beat will lead to an increased urine production. Some evidence to sustain this hypothesis is provided by Chaisemartin (1968). He observed in a related species, *Lymnaea limosa*, a reduction of the urine production and a decrease of the frequency of the heart beat after cauterization of both pleural ganglia. When these responses also occur in *Lymnaea stagnalis* after pleuralectomy they would account for the post-operative swelling reported by Hekstra & Lever (1960). It remains possible, however, that the secretory material of the DGC is not the only substance produced in the pleural ganglia which is involved in diuresis. Heart acceleration in *Lymnaea stagnalis* is known to be effected by a variety of chemicals (e.g., 5-hydroxytryptamine and dopamine) which are acting as neurotransmitters in molluscs (S.-Rózssa & Zs.-Nagy, 1967). Several types of neurotransmitter-like types of secretory granules were observed in the pleural ganglia during the present observations. Moreover, in a fluorescence histochemical study Sakharov & Zs.-Nagy (1968) demonstrated 5-hydroxytryptamine and dopamine in all central ganglia of *Lymnaea stagnalis*.

Another possible function of the DGC may be the regulation of the ion content of the blood. There is a substantial increase in the loss of ions from snails exposed to de-ionized water, due to diffusion through the skin and as a consequence of increased diuresis. The urine produced by *Lymnaea stagnalis*, although hypotonic to the blood, will be hypertonic to de-ionized water (cf. van Aardt, 1968). Therefore, exposure to the latter medium will stimulate the ion uptake mechanisms located in the skin, the kidney and, possibly, the intestinal duct (Greenaway, 1970). With regard to ion regulation it must be taken into account that the media used in the experiments were different in ionic composition, where tap water and the saline solution (NaCl in de-ionized water) are concerned. The changes described in the DGC of snails placed in the latter solution were, however, also observed in the DGC of animals exposed for 15 days in a NaCl solution in tap water of the same osmolality. No physiological experiments have so far been performed on the relation between the pleural ganglia and active ion transport. In *Lymnaea limosa*, however, cauterization of the pleural ganglia did not influence the sodium exchange of the snails. On the other hand, the sodium exchange was reduced after cauterization of the parietal ganglia (Chaisemartin, 1968). These ganglia and also the
visceral ganglion of *Lymnaea stagnalis* need further study from the point of view of osmotic and ionic regulation, because they contain another neurosecretory cell type (the Yellow Cells) which has been related to these processes (Wendelaar Bonga, 1970b).

V. SUMMARY

The neurosecretory cells present in the pleural ganglia of the fresh water snail *Lymnaea stagnalis*, the Dark Green Cells, were studied to establish whether these neurones are engaged in osmoregulation. Groups of 5 snails were exposed to conditions which induce changes in the diuretic activity (de-ionized water or a 0.1 M sodium chloride solution) for 1, 8, and 15 days, respectively. Animals kept in tap water were used as controls. The cell bodies as well as the axon terminals of the Dark Green Cells were studied quantitatively at the ultrastructural level. To estimate the rate of secretory activity of the neurones changes in the relative extent of the granular endoplasmic reticulum (determined by lineal analysis) and in the number of active Golgi zones were used as parameters. Lineal analysis was also used to determine the fractional volume of the mitochondria and of the cytosomes. Variations in the amount of secretory material in the cell bodies were determined by counting the number of elementary granules per surface unit. Accumulation and release of the secretory material in cross sections of the main neurohaemal area (the right pleuro-parietal connective) were determined by counting the total number of granule-containing axon profiles, and the number of these axons showing release phenomena, respectively.

Exposure to de-ionized water, presumably leading to enhanced diuresis, showed a statistically significant increase (a duplication in 15 days) of the extent of the granular endoplasmic reticulum and of the number of active Golgi zones per surface unit (2000 μ²). Moreover, a twofold increase of the volume of the cytoplasm per cell body was noted. In the axon terminals located in the right parieto-pleural connective an enhanced release of secretory material was observed. These trends, which were statistically significant, point to an activation of the Dark Green Cells when the animals are exposed to de-ionized water. During exposure of the snails to 0.1 M sodium chloride, a solution, which is hypertonic to the blood and known to reduce the rate of urine formation, the secretory activity of the DGC was markedly reduced. The observed decrease of the extent of the granular endoplasmic reticulum and of the number of active Golgi zones per surface area was statistically significant. At day 8 the occurrence of cellular atrophy was indicated by the presence of large numbers of newly formed lysosomes.
of autophagous nature. After two weeks the cytoplasmic volume per cell body was reduced to at least half the control value. In the axon terminals release of secretory material was hardly observed. The secretory material in the axon terminals, rapidly accumulated during the first hours of exposure, gradually diminished during the experiment which was partly due to release, and partly to lysosomal breakdown.

The changes observed in both experiments were reversible: the quantitative data obtained from snails kept in tap water for one week after a stay of 15 days in the experimental solutions was within the control range.

The results are in line with the hypothesis that the Dark Green Cells synthesize a substance which acts in diuresis. Additional physiological evidence is, however, needed to define more accurately the function of these neurones in the maintenance of the water balance.

ACKNOWLEDGEMENTS

The author is greatly indebted to Prof. Dr. J. Lever and to Dr. J. Joosse for their advice during the investigations, to Dr. H. H. Boer for his criticism during the preparation of the manuscript, to Dr. J. C. Jager for statistical advice, to Mr. T. Sminia, who performed the acid-phosphatase tests, and to Miss B. Plesch for correcting the English text.

This study was made possible by a grant of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

REFERENCES


BOER, H. H., 1965. A cytological and histochemical study of neurosecretory cells in


