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Formation, Storage, and Release of Neurosecretory Material Studied by Quantitative Electron Microscopy in the Fresh Water Snail Lymnaea stagnalis (L.)

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Summary. The process of neurosecretion in the fresh water snail Lymnaea stagnalis was analysed quantitatively at the ultrastructural level. Special attention was paid to the phloxinophilic neurones in the cerebral ganglia, the Caudo-Dorsal Cells (CDC).

The rates of synthesis, storage and release of neurosecretory elementary granules were studied in the CDC of animals fixed at time intervals of 4 hours, during a 24 hour period. The processes concerned show a diurnal rhythmicity. In the cell bodies synthetic activity, as determined by counting the number of active golgi zones per surface unit, is high during the night and low during the day. The reverse was found for the amount of secretory material (number of elementary granules per surface unit) stored in the cytoplasm. From these results it is concluded that also the rate of transport of the elementary granules through the axons fluctuates rhythmically. It has its maximum during the night and its minimum during the day. This conclusion was substantiated by the observation that the highest number of granules in the axons is found during the night.

The axon terminals of the CDC pass through four different stages during the 24 hour period. In axons being in the accumulation stage, which are found from the morning until the evening, the number of elementary granules increases rapidly. The neurohaemal zone increases in thickness during this process. In axons in the release stage, which predominate during the evening and the night, the contents of the elementary granules are extruded from the axon terminals, apparently by exocytosis. The rate of release of the secretory material into the body fluid, which is very low during the day, shows a rapid increase a few hours before sunset, and is probably correlated with the light intensity. A marked decrease of the amount of secretory material is noted in the neurohaemal area during the release stage. A daily turnover of nearly all secretory material accumulated in the axon terminals is suggested by the observations. Axons being in the reconstruction stage are mainly found during the night. They are distinguished by the presence of vesicular and tubular structures. These probably originate by fusion of microvesicles, which are considered to arise from the membranes of the elementary granules after exocytosis. After disappearance of the vesicles and tubules the axons enter the empty stage. This is frequently found in the night and in the early morning hours. In the morning the axon terminals enter the accumulation stage again.

Furthermore, accumulation and release phenomena were quantified in the axon terminals of 5 other neurosecretory cell types present in the ganglia. A diurnal pattern rather similar to that of the CDC was found in the Light Green Cells (the Medio- and Latero-Dorsal Cells in the cerebral ganglia). No rhythmicity was found in the neurohaemal areas of the four

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types of secretory neurones located in the ganglia of the visceral ring. A constant level of storage and release was established during the 24 hour period.

The relationship between synthesis, storage and release of the secretory material of the CDC is discussed.

**Key-Words:** Neurosecretory material—Lymnaea stagnalis—Formation—Release—Rhythmicity.

In many light microscope studies on neurosecretory systems of invertebrates (e.g., Highnam and Lusis, 1962; Rensing, 1966; Andrews, 1968) including the fresh water pulmonate snail Lymnaea stagnalis (Lever and Joosse, 1961; Joosse, 1964), the secretory activity of the neurones is regarded to be reflected by the amount of stainable material present in the cell bodies. The validity of this supposition has been questioned by Highnam (1965), who argued that no definite conclusions about the secretory activity can be drawn on the basis of the amount of secretory substance present in the cells, unless the rates of formation, transport, and release of the material are taken into account.

To study quantitatively these processes, several techniques have been applied, including autoradiography, cytophotometry and morphometrical methods. As far as invertebrates are concerned, the incorporation rate of labelled amino acids has been studied in only a few species (e.g., Bierbauer et al., 1967; Schooneveld, 1970). The amount of secretion stored in the neurones has been determined by using cytophotometry (Gersch and Wohlrabe, 1965; Rensing, 1966; Drawert, 1968) or, semiquantitatively, by means of an arbitrary scoring system (e.g., Siew, 1965; Kuhlmann and Nolte, 1966; Schooneveld, 1970). For these purposes the cells are usually stained with the Gomori techniques for neurosecretion. However, the selectivity of these techniques has been doubted (Simpson et al., 1966; Hadler et al., 1968), which reduces the value of these methods.

Morphometrical analysis in invertebrate neurosecretory systems has been limited to studies of changes in the volumes of cell bodies, of nuclei, or of nucleoli (Rensing, 1964, 1966; Gawande, 1969; Schooneveld, 1970). Such changes are known to reflect variations in the secretory activity. The vertebrate hypothalamic neurosecretory nuclei have also been studied in this way (Streefkerk, 1967; Olivereau, 1970).

However, a more direct approach in the morphometrical analysis of secretory processes is possible with the electron microscope, since at the ultrastructural level the cell organelles involved in the elaboration of the secretory material—the granular endoplasmic reticulum and the Golgi zones—as well as the secretory granules can be closely examined. Changes in the morphology (cf., Streefkerk, 1967; Pilgrim, 1969) as well as in the volumes of these cell constituents (Reinhardt et al., 1969a) after experimental stimulation have been reported for the hypothalamic neurosecretory nuclei of rats. Accordingly, quantitative investigations with the electron microscope seem to be the obvious histological way to study the relation between the various phases of the secretory process.

In a recent study of the neurosecretory cells of Lymnaea stagnalis (Wendelaar Bonga, 1970a), it appeared that the secretory material of at least two (the Caudo-Dorsal Cells and the Light Green Cells of the cerebral ganglion) out of the nine histochemically and ultrastructurally distinct types of neurosecretory cells is mainly released in the evening, indicating a 24 hour cycle. Because it was assumed
that this cyclical variation in the release activity is linked with activity changes within the cell bodies, a quantitative study of the ultrastructure of the cells and the axon terminals during the 24 hour period offers an opportunity to elucidate the relation between the formation, storage and release of neurosecretory material. For this study the Caudo-Dorsal Cells (CDC) were chosen, mainly because in the well defined neurohaemal zone of these cells, the periphery of the intercerebral commissure, almost no neurosecretory axons other than those of the CDC are present. In the neurohaemal zones of the Light Green Cells more than one type of axon terminals have been encountered (Wendelaar Bonga, 1970a).

It is well known that many physiological processes show a circadian rhythmicity (e.g., Aschoff, 1966). In *Lymnaea stagnalis* this was established for oviposition (Van der Steen, 1967; 1970). Since rhythmical processes may be under neuroendocrine control (e.g., Rensing, 1966), it seems of interest to study in some detail whether the other neurosecretory cell types present in *Lymnaea stagnalis* show a rhythmicity in their release activity. The release of neurosecretory material was therefore not only studied in the CDC, but also in five other cell types of which the location of the axon terminals is known (cf., Wendelaar Bonga, 1970a).

**Materials and Methods**

In June 1969 sexually mature specimens of *Lymnaea stagnalis* (L.), with a shell height of 30-32 mm, were taken from a population of about 300 adult animals, living under natural conditions in a concrete pond outside the laboratory. These snails had hatched in the previous summer. The reproductive period of this generation lasted from the end of April to the end of August, when most of the snails died.

Prior to fixation, the snails were examined with a dissection microscope for trematode parasites. Infected animals (less than 10%) were discarded.

For the main experiment groups of 10 snails were fixed at intervals of 4 hours during a 24 hour period, starting at 6.00 hr on June 19, 1969 (sunrise: 4.19 hr; sunset: 21.03 hr). During this period the water temperature at a depth of 15 cm varied between 18 and 22.5°C.

Additional observations were performed on 5 groups of 3 animals of the same population. They were fixed on June 24, with intervals of 1 hour, from 18.00 hr onwards (sunset: 21.04 hr; water temperature: 22°C). Furthermore, snails with a shell height of 29–32 mm, raised in the laboratory were examined. They had been kept under conditions described before (Wendelaar Bonga, 1970a) at a constant water temperature of 20±1°C and a daily photoperiod from 7.00 hr until 19.00 hr. After decapitation of the snails the central nervous systems and proximal parts of the nerves were dissected out and prepared for electron microscopy. The tissues were fixed for 2 hours in a veronal buffered (pH 7.4) mixture of glutaraldehyde (0.8%) and osmium tetroxide (1.0%) at 0°C. Postfixation followed for 30 minutes in a solution of uranyl nitrate (1.0%). After fixation and dehydration the nervous systems were cut into small parts, which were embedded in Epon 812. For quantitative determinations on the neurohaemal zones of *L. stagnalis*, cross sections were made of the intercerebral commissure, the right median lip nerve, the right parieto-pleural connective and the intestinal nerve. Of each of these structures 5 were selected from the available material, the criterion for selection being their diameter. This may greatly be influenced by the preparation procedure. For this purpose 2 μ thick cross sections were cut and measured by use of an eyepiece micrometer in the phase-contrast microscope. The diameters chosen were 175 ± 10 μ for the intercerebral commissure, 140 ± 10 μ for the right median lip nerve (measured at about 200 μ from its origin), 120 ± 20 μ for the right parieto-pleural connective and 90 ± 7 μ for the intestinal nerve (measured about 200 μ from its origin).

Ultrathin sections were cut on a Reichert ultramicrotome. They were stained with lead citrate and examined in a Zeiss EM9A electron microscope.
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**Observations**

**The Caudo-Dorsal Cells**

The CDC are located in two large groups in the caudo-dorsal part of the left and the right cerebral ganglion, near the origin of the intercerebral commissure. The left group consists of 20–40, the right group of 50–100 neurones. The length of the cell bodies varies considerably (from 15 to 80 μ), which is connected with the fact that the groups contain cells of at least three different degrees of polyplody (Boer, 1965). The secretory material produced by these neurones shows affinity for phloxin and azocarmine. It is transported via the axons which run from both cell groups towards the periphery of the intercerebral commissure, the neurohaemal zone of the CDC (Joosse, 1964). The ultrastructure of this neurosecretory system has been studied by Boer *et al.* (1968). The cells produce elementary granules with a mean diameter of 1500 Å. The mechanism of release of the contents of these granules has been studied before (Wendelaar Bonga, 1970a).

In the present study the cell bodies and axons of this neurosecretory system of 7 groups of 5 animals each, were investigated. The central ganglia of these groups were fixed at intervals of 4 hours, starting at 6.00 hr, during a period of 24 hours.

**Cell bodies of the CDC**

As the secretory material of the cells is primarily proteinaceous (Boer, 1965), it can be assumed that in particular the granular endoplasmic reticulum and the Golgi apparatus are involved in the formation of the secretory granules (cf., Beams and Kessel, 1963; Pilgrim, 1969). Accordingly, for studying variations in the rate of synthesis of the secretory product the morphology of the granular endoplasmic reticulum and of the Golgi zones was taken into account. Furthermore, the number of elementary granules present in the cytoplasm was studied, in order to obtain information about the amount of secretory material present in the cell bodies during the day.

For quantitative purposes, samples of the cytoplasm were obtained by cutting ultrathin sections of the right group of CDC. In order to reduce the effects of irregularities of the section thickness each sample consisted of 5 sections, cut at intervals of about 50 μ in planes perpendicular to the long axis of the neurones. Except in the region of the axon hillock, the cell organelles concerned were found to be evenly distributed throughout the cytoplasm. The axon hillocks were therefore excluded from the samples.

The endoplasmic reticulum is very extensive. Most of the lamellae are arranged in parallel arrays, forming large stacks, described as Nissl disks with the light microscope (Joosse, 1964). Bound ribosomes are numerous, while free ribosomes also occur, albeit in small numbers. Dilatations of the membranes (cisterns) are rarely found. They occur primarily in the areas adjacent to the Golgi zones. From initial investigations it was concluded that during the day no distinct morphological changes in the arrangement of the lamellae, or in the number and size of the cisterns, take place. Attention was therefore focussed on the Golgi apparatus and the elementary granules. From serial sections it appeared that the Golgi zones consist of 3–6 saccules, which measure 8–12 μ in length. The arrays of Golgi saccules often show a polarity. One side—the outer side when the saccules...
Fig. 1. "Active" Golgi zone in the cytoplasm of the cell body of a CDC. The distal parts of the Golgi sacculles contain accumulations of electron dense secretory material (arrows); large numbers of small clear vesicles (cv) and of elementary granules (eg) are present; some of the latter are relatively small and probably represent prosecretory granules; ts profiles of the tubular system; fixation: 22.00 hr

Fig. 2. "Inactive" Golgi zone in the cytoplasm of the cell body of a CDC. Secretory material is absent from the Golgi-sacculles (Gs); a few clear vesicles (cv) and elementary granules (eg) do occur; the granular endoplasmic reticulum (cr) shows elongate cisterns; fixation: 14.00 hr
are bent semicircularly—is normally turned to the granular endoplasmic reticulum. The membranes of both organelles show small evaginations at those places where they are nearest to each other. In this area small clear vesicles (⊙ 600–800 Å) are present. Similar structures have been described in many cell types and have been interpreted as indications for the transport of secretory material by means of the vesicles from the endoplasmic reticulum towards the Golgi saccules (cf., Beams and Kessel, 1968). Some saccules (1–3) at the inner side of the Golgi zones (occasionally also at the outer side) may contain electron dense material, which is evenly distributed between the lamellae or accumulated at their distal parts (Fig. 1). Obviously the elementary granules are formed by a process of budding. The granules in the Golgi zone are considered as still immature, because it has been demonstrated that the mean diameter of the elementary granules increases slightly in the period between their formation in the Golgi zones and their transport into the axons (Wendelaar Bonga, 1970a). In favourable sections a system of branching tubules is seen around the saccules and anastomosing with them. A similar structure has been described in the Golgi zones of other cell types (cf., Beams and Kessel, 1968), including the cells of the multifid gland of the pulmonate Helix pomatia (Ovtracht et al., 1970). Around the tubular system small clear vesicles (⊙ 600–900 Å) occur. Occasionally larger electron transparent vesicles (⊙ about 1000 Å) are found, characterized by a spiny outer membrane. These are known as coated vesicles in other cells, including vertebrate neurosecretory neurons (Friend and Farquhar, 1967; Pilgrim, 1969).

From the examination of a large number of Golgi zones in the cytoplasm of the CDC it appeared that, with regard to granule formation, two types can be distinguished.

1. “Active” Golgi zones (Fig. 1): in these zones electron dense secretory material occurs within the saccules, which may be dilated; near such Golgi zones “immature” elementary granules, occasionally with an irregular outer membrane, are generally found; clear vesicles are also present, as are profiles of the tubular system.

2. “Inactive” Golgi zones (Fig. 2): these lack electron dense secretory material in the saccules; the lamellae of the saccules are mostly lying tightly together; immature elementary granules are mostly absent in the Golgi areas; the clear vesicles are scarce.

For establishing whether changes occur in the secretory activity of the cells during the 24 hour period, attention was paid to the volume of the Golgi apparatus and to the number of active and inactive Golgi zones per surface unit. An increase of the activity of the Golgi apparatus may lead to an enlargement of its volume (cf., Pilgrim, 1969). Growth of the relative volume of the Golgi zones results in an increase of the number of Golgi profiles per surface unit in the tissue sections. To establish whether such an increase occurs in the CDC during the 24 hour period, the number of Golgi profiles per 5000 μ² of cytoplasm was determined. From the examination of 5 areas in different parts of the same cell group, it appeared that the number of Golgi zones per area varied by about 10% when the sampled areas were 5000 μ² each. The results presented in Fig. 3A show that there is little variation in the mean number of Golgi zones per surface unit between the experimental groups. It can therefore be concluded
that no obvious changes in the volume of the Golgi apparatus per volume of cytoplasm take place during the day.

The total number of active Golgi zones per surface unit is considered as a relative measure of the secretory activity in these neurones. For determining the variation of this activity during the 24 hour period, the number of profiles of active Golgi zones was counted in areas of 5000 μ² (the same as used before) of the cytoplasm of each animal of the experimental group. From Fig. 3A it appears that there is a tendency for the Golgi zones to be more actively engaged in granule synthesis during the night than during the day. From 6.00 hr onwards, the activity decreases. The lowest number (i.e., about 37% of the total number of Golgi profiles) of active Golgi areas was observed at 14.00 hr. A rise in the activity is already notable at 18.00 hr. The highest number of active Golgi zones (about 85% of the total number) was observed at 2.00 hr. The activity was still high at 6.00 hr.

In the areas of 5000 μ² selected for Golgi counts the number of elementary granules was also determined. Initial studies on 5 areas of this surface in one cell...
Fig. 4. Synaptic contact between an axon containing a neurotransmitter type of granules (ng) and a number of synaptic vesicles (sv), and an axon of a CDC (CDC) in the proximal part of the axonal tract; eg elementary granules.

Fig. 5. Accumulation of intertwined microtubules in an axon (probably of the CDC), located in the central part of the intercerebral commissure.
group showed that the numbers obtained for each area differed by less than 12\%, which indicates that the secretory material is fairly evenly distributed over the cells of the same group.

The mean granule densities per experimental group are presented in Fig. 3 B. A fluctuation during the day is apparent. In the morning, from 6.00 hr onwards, the amount of secretory material increases considerably. In this period the granules aggregate, forming large accumulations between the arrays of endoplasmic reticulum (Fig. 4). The accumulations are, however, distributed evenly in the cytoplasm. No conspicuous concentration of granules was observed in the axon hillock. The highest numbers of granules were found at 14.00 hr. In the evening, a sharp decrease in granule density is noticeable. The prominent accumulations disappear. The lowest numbers of granules were encountered at 22.00 and 2.00 hr (Fig. 5). During this period the granules are primarily located around the Golgi zones.

Comparison of Fig. 3 A and B shows that Golgi activity and granule density are inversely related. During the period when the highest numbers of active Golgi zones are found, the number of elementary granules diminishes considerably. This relation seems to be contradictory, unless discharge of the elementary granules into the axons is taken into account.

The Axons

The axons of the CDC run as a thick bundle from the cell bodies towards the intercerebral commissure. They branch frequently, even in their proximal parts. A fair number of them ends in the periphery of the cerebral ganglia, near the commissure. The majority of the axons, however, enters the commissure: in cross sections several thousands of small axons (\( \vartheta 0.2-2 \mu \)) were observed in its central parts. Most of these axons contain elementary granules of the type characteristic for the CDC. The periphery of the commissure is clearly delimitated from the central area by the presence of numerous large axon endings (\( \vartheta 1-10 \mu \)) containing neurosecretory elementary granules. This periphery constitutes the neurohaemal area. The axon endings are distributed fairly evenly over the total surface of the commissure. Most of them originate from the CDC, while a low percentage of the axons contain granules typical for the Light Green Cells (cf., Wendelaar Bonga, 1970a). In all parts of the commissure axons containing small dense cored vesicles (\( \vartheta 800-1200 \ \text{Å} \)), characteristic for neurotransmitters, occur. Occasionally synapses were found on axons of the CDC (Fig. 6). Prominent membrane differentiations are lacking in the synaptic regions, as is common for gastropod synapses (cf., Wendelaar Bonga, 1970a). In the axons large numbers of microtubules are found. Generally they form straight and parallel bundles, but sometimes intertwined accumulations occur (Fig. 7), similar to those observed by Reinhardt (1969 b) in the neurohypophysis of the rat.

To study the dynamics of the secretion process in the axons and axon terminals, attention was paid to the transport, storage and release of the elementary granules. The two latter processes, which take place in the neurohaemal zone, were investigated quantitatively.

Unfortunately, the rate of transport of elementary granules through the axons cannot be studied directly with the methods used. Nevertheless, a study of cross sections of the axonal tract near the origin of the intercerebral commissure
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Fig. 8 A and B. Determinations in the neurohaemal zone of the CDC, the intercerebral commissure, of 7 groups of 5 animals, fixed at different times during the 24 hour period. Per animal 3 cross sections of the intercerebral commissure were examined. The horizontal black bars indicate the period between sunset and sunrise. A Black dots: total number of axon profiles containing elementary granules; open circles: number of granule containing axon profiles facing the perineurium; B black dots (left scale): number of axon profiles showing release phenomena; broken line (right scale): light intensity. Each dot represents the mean number per group ($\pm$ S.E.)

(2 animals per group were studied) indicated that transport is a phasic process with a daily periodicity. This was concluded because in these sections the highest numbers of elementary granules were present between 22.00 and 10.00 hr, whereas the lowest numbers were found at 14.00 and 18.00 hr. The observation that an increase of the number of elementary granules occurs during the night and the morning, is in accordance with the low granule density observed in the cell bodies during this period.

For estimating the total amount of neurosecretory material of the CDC present in the neurohaemal area, the number of profiles of axons and axon endings containing elementary granules of the CDC type was taken as a parameter. The total number present per animal in the peripheral zones of three cross sections was counted. These sections were always cut from the same areas of the commissure, viz., one in the middle and the two others at about 200 $\mu$ from the left and right ganglion, respectively. The results of the counts on the experimental groups are presented in Fig. 8A. From 6.00 hr onwards an increase of the number of granule filled axons takes place. Highest numbers were found at 14.00 and 18.00 hr. A decrease to about 25% of the maximal value takes place during the evening and the night. This data suggests that there is a daily turnover of at least 75% of the neurohormone present in the neurohaemal zone at 18.00 hr. The mean quantity per animal present at this time in the intercerebral commissure was calculated on
the basis of the number of granule filled axon terminals, the mean size of the intercerebral commissure, and the mean total volume of the elementary granules in the axons. The latter was determined by lineal analysis (Loud et al., 1965) of 2 samples (100 $\mu^2$ each) per animal. The mean volume of the elementary granules present in the neurohaemal zone at 18.00 hr amounts to $2.3-2.9 \times 10^6 \mu^3$ per animal. This means that the volume of the total number of elementary granules released (75%) equals the total volume of cytoplasm of all cell bodies of the CDC ($2.1 \times 10^6 \mu^3$ per animal) as calculated from data presented by Boer (1965).

A daily turnover of nearly all neurosecretory material in the neurohaemal zone of the CDC does not occur throughout the year. Preliminary determinations on groups of snails fixed in October 1969, when the water temperature was lower (13-16°C) than in June (18-22.5°C), showed that in these animals only a small part (20-35%) of the material present is released during the evening and night.

The maximal number of profiles of axons and axon terminals per cross section of the intercerebral commissure is more than 600. From studying serial sections it was estimated that at least 35% of them are profiles of axon terminals, i.e., about 200 per cross section. From this figure and from the size of the intercerebral commissure it was assessed that the neurohaemal area contains more than 120,000 axon terminals. As the mean number of neurosecretory neurones in both groups of CDC is less than 150 (Joosse, 1964), the mean number of neurosecretory axon endings per neuron is more than 800.

The release of neurosecretory material from axon terminals in *L. stagnalis* is regarded to take place by exocytosis (Wendelaar Bonga, 1970a). Phenomena indicating exocytosis are the occurrence of omega-shaped indentations in the outer axonal membrane, the occasional presence in these indentations of electron dense material of the same appearance as the contents of the elementary granules, and the presence of clusters of microvesicles in the axon terminals.

For establishing the rate of release in the neurohaemal area, the number of axon profiles showing release phenomena was determined in three cross sections of each of the intercerebral commissures of the animals of the experimental groups (Fig. 8 B). During almost the total length of the period of light (from 6.00 till 14.00 hr) release occurs at a low rate, viz., in less than 1% of the granule laden axons. There is a slight increase of the release activity (to about 2%) at 18.00 hr. In the evening a steep rise to about 25% of the granule containing axon profiles at 22.00 hr is evident. During the rest of the period of darkness release activity remains high (16% at 2.00 hr). Then it falls rapidly to less than 0.5% at 6.00 hr. Thus, release of the secretory product from the neurohaemal area takes place nearly exclusively during the night. This conclusion is consistent with the observed decrease of the number of elementary granules in the axon terminals during the same period (Fig. 8 A).

Release phenomena in *L. stagnalis* occur exclusively in axon terminals facing the perineurium. Granule extrusion into the interaxonal space as observed in the neurohaemal organs of several insect species (e.g., Scharrer, 1968; Normann, 1970) was not found. Initial observations suggested that the number of granule filled axons facing the perineurium varies during the day. Thus, it might be possible that the rate of release is a function of the number of axons facing the perineurium. To investigate whether such a relation exists, this number was
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Fig. 9. Part of the periphery of the intercerebral commissure showing a large number of profiles of axon terminals (ax) of the CDC, most of which contain moderate numbers of elementary granules; *nu* nucleus of glial cell; *gl* glial cell processes; *pe* perineurium; fixation: 14.00 hr

determined. Fig. 8A shows that it increases rapidly from 6.00 until 14.00 hr and then decreases during the rest of the 24 hour period. The shape of the curve is quite different from that for the rate of release. Thus it can be concluded that
the presumed relation does not exist. On the other hand, the curve parallels that for the total number of granule filled axons in the neurohaemal zone (Fig. 8A). It is therefore indicated that increased storage of neurosecretory material induces the axons to penetrate in large numbers through the layer of filamentous glial cells, which normally separates the axons from the perineurium (cf., Wendelaar Bonga, 1970a), as is consistent with the electron microscopic observations (Figs. 9 and 12).

The period of increased release activity starts at the beginning of the evening, suggesting that there is a relation between the start of the release and the light intensity. For a further study of this possible relationship 5 groups of 3 animals grown in the laboratory at a constant water temperature ($20 \pm 1^\circ C$) and at a light period of 12 hours (7.00–19.00 hr) were examined. These groups were fixed with intervals of one hour, starting 2 hours before the end of the light period. The percentage per animal of those axon profiles facing the perineurium which showed release phenomena increased from 7–25% before and at the moment the light was switched off, towards 50–80% within two hours thereafter.

These results suggest that light may be an important factor in the regulation of the release activity.

The changes occurring in the axon terminals during the day will now be described on the basis of the quantitative results presented in the previous sections and on the close observation at the ultrastructural level of the neurohaemal areas of the groups of snails used in the experiments. For additional information about the morphological changes in the axon terminals of the CDC around sunset 5 groups of 3 animals, living under natural conditions and fixed at intervals of one hour (start: 18.00 hr, sunset: 21.04 hr) were examined.

Four stages are distinguished in the axon terminals: a) accumulation stage; b) release stage; c) reconstruction stage; d) empty stage.

a) Accumulation Stage. In this stage the number of elementary granules in the axon endings increases rapidly. This accumulation is evident from the increase in the total number of granule containing axon profiles in cross sections of the intercerebral commissure (Fig. 8A). The rise of the number of granules leads to an enlargement of the axon terminals. As a result of this phenomenon the thickness of the layer of neurohaemal tissue around the commissure increases from 4–8 $\mu m$, at the start of the accumulation stage (around 6.00 hr), to 10–15 $\mu m$ at the time when accumulation is completed (between 14.00 and 18.00 hr, cf., Fig. 8A). The layer of processes of filamentous glial cells at the surface of the commissure, which is rather extensive at 6.00 hr (Fig. 12) is progressively pushed aside by axons crowded with elementary granules (Fig. 9). Occasionally, small groups of axons penetrate into the perineurium, giving rise to small nerves, which are considered as extensions of the neurohaemal area (Wendelaar Bonga, 1970a).

b) Release Stage. The entrance into the release stage of granule filled axon endings facing the perineurium becomes apparent firstly from the decrease of the granule diameter (Figs. 10a, 11) and the appearance of clusters of microvesicles (Fig. 10b) and of omega-shaped indentations (Fig. 10c). The decrease of the granule diameter may result in a size gradient of the granules towards the axonal membrane. Such a gradient may be absent, as for instance in the small
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Fig. 10a–d. Several successive phases in the process of release of the contents of the elementary granules from the axons of the CDC. Bars represent 1 micron. a Axon terminals containing elementary granules of normal and decreased size. The small granules (arrows) are primarily located near the axonal membranes facing the perineurium (pe); fixation: 19.00 hr. b Indication of exocytosis. Contents of elementary granules of relatively small size are present outside the axon terminal, within invaginations of the axonal membrane (arrows); fixation: 19.00 hr. c Groups of small clear vesicles (cv) are present in the area adjacent to the perineurium. The axonal membrane shows small indentations (arrows); fixation: 19.00 hr. d Membrane bounded, irregular shaped vesicular and tubular structures are present in an axon terminal devoid of elementary granules; fixation: 22.00 hr
Fig. 11 A and B. Distribution of the diameters (in Å.U.) of the profiles of elementary granules (open bars, left scale; 1000 measurements per histogram) and of profiles of clear vesicles (mottled bars, right scale; 230 measurements) in axon terminals of the CDC, located adjacent to the perineurium in the intercerebral commissure. A Determinations in non-releasing axon terminals; fixation: 14.00. B Determinations in axons being in the first phases of release (cf., Fig. 10a–c); fixation: 19.00 hr

number of axons showing release during the period of light. Clusters of microvesicles are more frequently observed than omega-shaped indentations, probably because the indentations are more transient than the vesicles (cf., Normann, 1970). The release phenomena mentioned are primarily found from 18.00 to 2.00 hr. They predominate in the first hours of this period (from 19.00-21.00 hr). It was observed that during the period of release the number of elementary granules in the axon terminals falls rapidly, as was already indicated by the decrease of the number of granule filled axon profiles in the neurohaemal zone (Fig. 8 A). Also the volume of the axon terminals diminishes. As a consequence the thickness of the neurohaemal layer decreases.

c) Reconstruction Stage. From 20.00 hr onwards tubular and vesicular structures are present in a growing number of axons. They appear at first in the axon terminals facing the perineurium after release of part of the secretory material. Later on, they are also found in axons devoid of elementary granules at a distance from the perineurium. At 2.00 hr they occur in the majority of the axon profiles. At this time they may occupy the axon terminals completely (Fig. 10d).

The vesicular structures, which have electron transparent contents, are bounded by an irregular membrane. They appear in those areas of the axoplasm where release takes place. Their morphology (see also Fig. 14) suggests that they are formed by fusion of microvesicles. This is also indicated by the fact that the microvesicles disappear from the axon terminals at the time when the vesicular structures become visible. The tubules are more prominent than the vesicular structures. Their diameter (200–800 Å) is usually larger than that of microtubules (∅ 200 Å), which occur in more proximal parts of the axons. The tubules are structurally different from microtubules in that they are limited by a membrane,
Fig. 12. Part of the periphery of the intercerebral commissure with a large number of profiles of axons of the CDC (ax), most of which are devoid of elementary granules; arrow: axon containing a few number of elementary granules; nu nucleus of glial cell; gl processes of glial cells which are extended at the surface of the commissure, separating the axons from the perineurium (pe); fixation: 6.00 hr

Fig. 13. Multilamellar bodies in an axon of a CDC. In the lower body the bilamellate structure of the concentrically arranged membranes is visible, as are a large number of small vesicles (v). The profile of the upper body only shows the vesicular structures; eg elementary granules of the CDC type

Fig. 14. Part of an axon terminal, fixed during the release stage, of a Light Yellow Cell. The axon terminal is located at the periphery of the intestinal nerve; arrows: membrane indentations; cv cluster of clear vesicles; vt vesicular and tubular structures; fixation: 10.00 hr
whereas the microtubules consist of helically arranged fibrils. The tubular structures often interdigitate. It is assumed that they arise by elongation of the vesicular structures.

At 6.00 hr the tubular and vesicular structures have disappeared from nearly all axon terminals.

d) Empty Stage. Starting at 22.00 hr, an increasing number of empty axon terminals is encountered. They are devoid of elementary granules and of tubular and vesicular structures. In these axons only the ordinary axonal organelles, microtubules, mitochondria and an occasional cytosome, occur. Empty axon terminals do not face the perineurium, but are separated from it by granule filled axons or extensions of filamentous glial cells (Fig. 12). At 6.00 hr about 75% of the previously filled axon terminals is empty. At 10.00 hr most of them have again started to accumulate elementary granules.

Each of the stages dominates during a distinct period of the day. This means that every 24 hours the axon terminals pass through the four stages. The axons facing the perineurium enter the release stage rather synchronously: between 18.00 and 20.00 hr the mean percentage of them showing release increases from about 7 to 85. The first axons in the third stage were found at 20.00 hr, indicating that it probably takes less than 2 hours for an axon to release its secretory material (cf., Wendelaar Bonga, 1970b). However, it appeared that at 2.00 hr the percentage of releasing axons at the surface of the commissure is still about equal to that at 20.00 hr. This indicates that axons that have released their granule content are replaced at the surface of the commissure by nerve endings still crowded by elementary granules. The fact that empty axons are always located at a certain distance from the perineurium supports this view. This mechanism may account for the large periods of overlap of the different stages. Due to the presence of these periods of overlap it is impossible to determine accurately the total amount of secretory material released every day. The morphological data presented suggest, however, that the contents of each axon terminal are released every 24 hour. This implicates that the daily turnover of the secretory material is probably more than 75% of the amount present in the neurohaemal area at the start of the release period. A total turnover is suggested.

In a number of species multilamellar bodies (cf., Bohm and Parker, 1968) have been described in the axons of neurosecretory neurones. Knowles (1964) suggested that these structures are engaged in the synthesis of neurohormones. Normann (1969) correlated their presence with the release of these substances. In the axons of the CDC multilamellar bodies were only occasionally found, viz., in less than 0.5% of the axon profiles. They consist of a series of concentrically arranged paired lamellae, which show dilatations in the central area and at the periphery of the bodies. The bodies are surrounded by clear vesicles of varying size (Fig. 13). Neither within the lamellae, nor in the vesicles were electron dense contents observed. The number of the bodies did not vary obviously during the 24 hour period. They were found in all parts of the axons of neurosecretory as well as of ordinary neurones. No indications that they are involved in the formation of granules were obtained.

The multilamellar bodies should not be confused with the lamellate type of cytosome which has been described in the neurones of many species, including
Fig. 15A and B. Determinations in the neurohaemal zone of the Light Green Cells, the median lip nerve, of 7 groups of 5 animals, fixed at different times during the 24 hour period. Per animal 3 cross sections of the right median lip nerve were examined. The horizontal black bars indicate the period between sunset and sunrise. A black dots: total number of axon profiles containing elementary granules; open circles: number of granule containing axon profiles facing the perineurium; B number of axon profiles showing release phenomena.

Each dot represents the mean number per group (± S.E.)

gastropods (Nolte et al., 1965). These structures, which are limited by a unit membrane, were also found in the axons of the CDC. Their number, like that of the multilamellar bodies, is very low.

Other Types of Neurosecretory Cells

In addition to that of the CDC, the location of the neurohaemal areas of 5 other neurosecretory cell types is known. On the basis of the staining results obtained with the Alcian Blue/Alcian Yellow technique, these neurones have been called the Light Green Cells, the Dark Green Cells, the Yellow Green Cells, the Light Yellow Cells and the Yellow Cells (Wendelaar Bonga, 1970a). The first type occurs in the cerebral ganglia. The other types are found in the visceral ring, which consists of the paired pleural and parietal ganglia and the single visceral ganglion.

Storage and release of neurosecretory material was quantitatively investigated in the neurohaemal zones of these cells by the same methods and in the same groups of animals as used for studying the CDC.

Two large groups of Light Green Cells are located in the medio- and latero-dorsal parts of each of the cerebral ganglia. The secretory product of these cell groups, originally called the Medio- and Latero-Dorsal Cells, is transported by large axonal tracts towards the median lip nerves (Joosse, 1964). Axon terminals are located peripherally over the entire length of these nerves. They contain elemen-
Fig. 10. Determinations in the neurohaemal zones of four neurosecretory cell types located in the visceral ring, of 7 groups of 5 animals, fixed at different times during the 24 hour period. Per animal 3 cross sections of the right pleuro-parietal connective, neurohaemal zone of the Dark Green Cells (DGC), and of the intestinal nerve, neurohaemal zone of the Light Yellow Cells, the Yellow Green Cells (YGC) and of the Yellow Cells (YC), were examined. The horizontal black bars indicate the period between sunset and sunrise. Black dots: total number of axon profiles containing elementary granules; open circles: number of axon profiles showing release phenomena.

Fig. 16. Determinations in the neurohaemal zones of four neurosecretory cell types located in the visceral ring, of 7 groups of 5 animals, fixed at different times during the 24 hour period. Per animal 3 cross sections of the right pleuro-parietal connective, neurohaemal zone of the Dark Green Cells (DGC), and of the intestinal nerve, neurohaemal zone of the Light Yellow Cells, the Yellow Green Cells (YGC) and of the Yellow Cells (YC), were examined. The horizontal black bars indicate the period between sunset and sunrise. Black dots: total number of axon profiles containing elementary granules; open circles: number of axon profiles showing release phenomena.

Fig. 15A. A variation, suggesting a diurnal rhythmicity, is evident in the mean number of granule filled axon profiles as well as in the mean number of axon profiles facing the perineurium (Fig. 15A), and in the mean number of axon profiles showing release phenomena (Fig. 15B). The number of granule filled axons is very...
low at 10.00 hr. At 14.00 hr it has increased more than fourfold. Then it decreases gradually. The release activity is low from 6.00 till 14.00 hr; at 18.00 and 22.00 hr it is high and then it levels off. As in the intercerebral commissure four stages were distinguished in the morphology of the axons in the median lip nerve: accumulation stage (from 10.00 till 14.00 hr), release stage (from 18.00 till 2.00 hr), reconstruction stage (from 18.00 till 6.00 hr) and empty stage (from 22.00 till 10.00 hr) do exist. Thus the Light Green Cells resemble the CDC as far as the variations in storage and release of the secretory material, as well as the morphological changes during the 24 hour period are concerned. Only slight differences were found. In the intercerebral commissure the lowest number of granule filled axons was found at 6.00 hr, in the lip nerves four hours later. At 18.00 hr the release activity is low in the commissure, while it has already reached a high level in the lip nerves.

The four types of neurosecretory neurones of the visceral ring, the Yellow Green Cells, the Dark Green Cells, the Light Yellow Cells and the Yellow Cells are located in several small groups in the visceral ring. Their axons do not form major axonal tracts but run individually or in small bundles to the neurohaemal zones. These are very extensive and include the peripheries of the connectives and nerves of the ring, as well as large areas of the perineurium. The axon terminals of the Dark Green Cells are mainly distributed around the pleural ganglia. The axon endings of the other cell types occur intermingled in all the areas mentioned (Wendelaar Bonga, 1970a).

For quantitative determinations areas where the axon terminals are most numerous were selected. The secretory material of the Dark Green Cells was studied in 3 cross sections, 100 μ apart, of the connective between the right pleural and parietal ganglion. For studying the material of the Yellow Green, Light Yellow, and Yellow Cells, 3 cross sections were cut of each of the intestinal nerves, at distances of about 200, 400, and 600 μ from its origin in the visceral ganglion, respectively. The different types of axon terminals can easily be distinguished because of the characteristic appearance of the elementary granules they contain. In only a small number of cases (less than 5%) was identification not possible, viz., in almost empty axons at the end of the release period, due to the changes in size of the granules which occur just prior to exocytosis.

The results of the counts performed on the axon terminals of the four types of neurosecretory cells in the visceral ring are presented in Fig. 16. It appears that storage (number of axon profiles with elementary granules) and release (number of terminals showing release phenomena) of the secretory material is fairly constant during the day in all types of neurones. The observation that axons in the stages of accumulation and reconstruction (Fig. 14) are found throughout the 24 hour period in about the same numbers supports this conclusion.

**Discussion**

Quantitative investigations at the ultrastructural level are rather scarce. Recent studies by Reinhardt et al. (1969a, b) are the first dealing with a morphometrical analysis of the ultrastructure of the neuroendocrine cells. These authors studied the effects of dehydration on the hypothalamo-hypophysial system.
of the rat, by utilizing the lineal integration technique. This technique, yielding the relative volumes of cell organelles, is, however, rather laborious, as a very large number of high power micrographs have to be prepared, especially for studying widely dispersed organelles. For this reason in the present study density counts were preferred, the more so since the objects studied—Golgi zones and elementary granules—are distinct units which are easily recognized on low power electron micrographs.

Cell Bodies of the CDC: Synthesis and Storage

In the hypothalamic neurosecretory nuclei of vertebrates experimental stimulation of the hormone synthesis is known to result in an increase of the number and size of the cisterns of the granular endoplasmic reticulum, and of the number of free ribosomes in the cytoplasm (Nemetschek-Gansler, 1965; Streefkerk, 1967). Activation of neurosecretory cells in the annelid worm Enchytraeus albidus (Gersch and Ude, 1967) also resulted in an increase of the extent of the cisterns. Cymborovski and Dutkowski (1969), who established a cyclical activity pattern in the secretory process in neurosecretory neurones of the cricket Acheta domesticus, observed a daily variation in the cytoplasmic basophilia, indicating a varying content of RNA. Contrary to these findings, no obvious morphological changes occur during the day in the ER of the CDC of Lymnaea stagnalis. This conclusion seems valid: since cisterns of the granular endoplasmic reticulum of the CDC are very scarce, any increase in their number would have been noticed. Apparently, the morphology of the endoplasmic reticulum does not reflect the rate of synthetic activity in these cells. Gomez Dumm and Echave Llanos (1970), who found a daily fluctuation in the Golgi activity of the somatotropic cells of the mouse pituitary, also failed to show any obvious change in the morphology of the endoplasmic reticulum.

A cyclical pattern was found in the relative number of active Golgi zones. The criterion used in this study for Golgi activity, viz., the presence of secretory material within the Golgi saccules, seems justified, since in a large number of neurosecretory as well as of other cell types the presence of secretory material within the Golgi lamellae has been positively correlated with secretory activity (Beams and Kessel, 1969). It has to be stressed that the adjective “active” as used in this study, refers only to the formation of elementary granules. “Inactive” Golgi zones may be actively engaged in other cell functions, as is for example indicated by the occurrence of coated vesicles which are regarded as stages in lysosome formation (Friend and Farquhar, 1967; Pilgrim, 1969). The increased activity of the hypothalamic neurosecretory nuclei induced by stress is expressed at the ultralevel by the appearance of large Golgi cisterns containing secretory material resulting in an increased rate of formation of elementary granules (Nemetschek-Gansler, 1965; Streefkerk, 1967). Furthermore, a simultaneous rise in the enzymatic activity of the Golgi apparatus has been demonstrated (e.g., Jongkind and Swaab, 1970).

Daily changes in the Golgi morphology have up till now only been noted by Gomez Dumm and Echave Llanos (1970) in the mouse somatotrophic cells. At noon and in the evening numerous vacuoles, vesicles and stages of granule
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formation occurs. During the other periods of the day the Golgi zones are small and well defined, lacking signs of secretory activity.

The maximal number (at 2.00 hr) of active Golgi zones per surface area in the CDC of *Lymnaea stagnalis* amounts to twice the minimal number (at 14.00 hr). Because the number of Golgi profiles per surface unit is constant during the day, it can be concluded that the number of granules formed per unit of plasma volume may also vary by a factor of two. However, when the total production of the cells of maximal and minimal activity is considered, this factor cannot directly be applied because changes of the cell volume are not taken into account. There is much evidence that considerable changes in the cell volume occur when the secretory activity varies. Such changes will be reflected in the total amount of secretory material produced. A positive relation between the secretory activity and cell volume has been established in the hypothalamic neurosecretory centres in vertebrates (Streefkerk, 1967; Olivereau, 1970; Reinhardt et al., 1969a). In several invertebrate species volume differences of neurosecretory cells have been used as a measure for the secretory activity of these cells (Brady, 1967a, b; Siew, 1965; Schooneveld, 1970). In *Drosophila melanogaster* a circadian rhythm with a circadian rhythmcity was observed in the volume of some neurosecretory cell bodies (Rensing, 1964; 1965). In most of the neurosecretory cell groups of *Lymnaea stagnalis*, including the CDC, changes of the cell volumes cannot be established, due to the presence of various size classes of these cells within one group. These size classes have been related to different degrees of polyploidy (Boer, 1965). If it is assumed that also in *Lymnaea stagnalis* the cytoplasm is enlarged with increasing cell activity, then the above estimated difference in production rates at minimal and maximal activity (a factor of two) is too low.

The most striking daily fluctuation occurring in the cell bodies of the CDC regards the number of elementary granules present in the cytoplasm. The rate of accumulation and discharge of the secretory granules varies synchronously in the cells of one group. This finding is consistent with the observation of Joosse (1964), that in a given specimen the secretory material is equally dense in different cells of both groups of CDC. These observations are in contrast with the results of many authors, who found large differences in the amounts of secretory material in the cell bodies of neurones of the same type (e.g., Mordue, 1967). The observed synchrony in the CDC makes these cells a favourable object for studying the relations between the secretory processes.

An inverse relationship between the amount of secretory material and the synthetic activity, as found in the cell bodies of the CDC, has also been reported for the rhythmical neurosecretory cells in the cricket *Acheta domesticus* (Cymborovski and Dutkowski, 1970). In this species maximal amounts of aminoacids were incorporated when the affinity of the cytoplasm for neurosecretory stains was minimal. A similar relation is indicated by the investigations on neurosecretory cells in the oligochaete *Enchytraeus albidas* (Gersch and Ude, 1967) and in the rat (Streefkerk, 1967). In the initial period of experimental stimulation of these cells a decrease of the numbers of elementary granules was noted in the cell bodies.

From the observed changes in the rate of Golgi activity and the variation in the granule content in the cell bodies of the CDC an inference can be made about the relative numbers of elementary granules transported at different times.
during the 24 hour period. Accumulation of elementary granules will occur whenever the rate of formation exceeds the rate of discharge into the axons. Consequently, transport must be very low during the day, when Golgi activity is minimal, since in this period accumulation takes place in the cell bodies. On the other hand, the rate of transport has evidently increased greatly during the evening and the night, as the granule density decreases significantly in this period, although the Golgi activity has increased to twice that during the day.

**Axon Terminals of the CDC: Release**

Morphological evidence for exocytosis as the mechanism of release of the contents of the granules in *Lymnaea stagnalis* has been presented before (Wendelaar Bonga, 1970a). In this study additional information was obtained about the details of the process of release. The observed diminution of the size of the granules prior to exocytosis, apparently reflects a change in the internal organization of the granules just before release. The remarkably high incidence of artifacts in the morphology of the granules in releasing axons after inadequate fixation confirms this view (Nolte, 1967; Wendelaar Bonga, 1970a).

Not only the absence of morphological evidence, but also the finding that the number of small clear vesicles is not proportional to the number of elementary granules of decreased size, suggests that the clear vesicles do not arise by budding from elementary granules before release. Evidence for this way of formation was obtained in blattarian insects by Scharrer (1968). The mechanism accounting for the decrease of the size of the granules remains obscure. As in some invertebrate species (cf., Normann, 1970) and also in the axon terminals of the neurohypophysis of some vertebrate species morphological evidence for exocytosis has been presented (Bunt, 1969; Nagasawa et al., 1970). On the other hand, biochemical findings on vertebrates indicating that the ratio between the neurohormone and the carrier protein in the intact granule differs from that found after release into the blood, has recently been used as an argument that exocytosis probably is not the main process of release (Thorn, 1970). However, the concept of exocytosis as the mechanism of release does not necessarily imply that the granular contents reach the blood unmodified. The observed changes in granule morphology in releasing axons in *Lymnaea stagnalis* as well as in other species (e.g., Shivers, 1969) are indications that a transformation of the secretory material takes place prior to exocytosis.

In *Lymnaea stagnalis* the appearance of clear vesicles coincides with the occurrence of omega-shaped figures. The omega-shaped figures are less numerous than the vesicles. These observations are in agreement with the results of Normann (1970) in the corpora cardiaca of the blowfly *Calliphora erythrocephala*. In this species a positive correlation between the frequency of the vesicles and the omega-shaped figures was found during release provoked by electrical stimulation. The higher incidence of the clusters of vesicles suggested that they persist longer than the omega-shaped figures. The observations in *Lymnaea* support the suggestion of Normann (1969) that, after exocytosis, the clear vesicles are formed from remnants of the membranes of the elementary granules.

Tubular and vesicular structures, as found in the axon terminals of the CDC after release of the contents of the elementary granules, have been reported in
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a few other cases, viz., in axon terminals in the neurohaemal region of the median eminence of toads (Rodriguez, 1969) and in the neurohypophysis of young rats (Vollrath, 1969). The hypothesis that these structures in *Lymnaea* originate by fusion of clear vesicles, and, therefore, represent the membranous material of the elementary granules, accounts for the disappearance of the microvesicles at the time when the structures arise. The reverse possibility, i.e., that the vesicles arise from the tubules, has been considered by Rodriguez (1969) and Vollrath (1969). However, in these studies the course of this process with time was not investigated.

The fate of the tubular structures in *Lymnaea stagnalis* is not clear. When they have disappeared, the axon terminals contain only microtubules and mitochondria. Autophagous digestion of the tubules in the distal parts of the axons seems unlikely, since lysosomes do not generally occur in these areas. Multilamellar bodies are also extremely scarce. The suggestion of Normann (1969), based on studies on *Calliphora erythrocephala*, that the small vesicles are transformed into multilamellar bodies is therefore not supported by the results obtained in the present study.

**Relation between Formation, Storage, and Release**

In light microscope studies the rate of secretory activity of invertebrate neurosecretory systems has often been correlated with the amount and the distribution of stainable material in the neurosecretory cells. In early literature, a high secretory activity was attributed to cells containing large amounts of secretory material in the cell bodies (e.g., Dupont Raabe, 1956; Fraser, 1959). However, more recently several authors (Highnam and Lusis, 1962; Liotti and Rosi, 1968; Andrews, 1968) regarded such cells as inactive, because in their opinion accumulation of secretory material in the cell bodies would indicate that release is limited. In this concept a low amount of material present in the cell bodies is indicative for an increased activity, especially when there is much secretory material in the axons. For *Lymnaea stagnalis* the same assumption was made by Lever and Joosse (1961) in a study of the lateral lobe, and by Joosse (1964), in an investigation of the year cycle of the CDC. This hypothesis remains speculative — as was discussed by Highnam (1965) — as long as data about the quantitative relationship between formation and release of the neurosecretory material is lacking. In the present study such information became available by the quantitative analysis of the CDC during the daily cycle. The results confirm the supposition that a low amount of neurosecretory material in the cell bodies reflects a high rate of release.

In invertebrates daily changes in neurosecretory cells have especially been reported for insects. In these animals several functions and processes (e.g., locomotion, metabolism) show a diurnal periodicity. They may well be under neuroendocrine control, as in some cases neurosecretory cells show parallel rhythms, although no definite relationships have been demonstrated (Rensing, 1965; Brady, 1965b; Cymborovsky and Dutkowsky, 1969).

In the gastropods the information on diurnal variations of neurosecretory cells and of body functions is limited. Gorf (1963) established that the number of neurones containing secretory material in the central ganglia of *Vivipara vivipara* varied during the 24 hour period, as was also observed in *Helix pomatia* (Jungstand, 1962). In *Aplysia californica* a circadian rhythmicity was shown in the
spike activity of a neuron (Strumwasser, 1965). This cell is presumed to be neurosecretory (Frazier et al., 1967). Diurnal rhythms have been reported in oxygen consumption of Littorina littorea and Urosalpinx cinerea (Sandeen et al., 1954) and in the locomotory activity of Oxychilus cellarius (Tercafs, 1961), Nassarius obsoleta (Webb et al., 1959) and Aplysia californica (Kupfermann, 1968). In several pulmonate snails oviposition occurs mainly during the night (Van der Steen, 1967). However, neuroendocrine relationships have so far not been established.

The function of one of the rhythmically active cell types in Lymnaea stagnalis, the LGC, has been studied by Joosse and Geraerts (1969). They obtained evidence that cauterization of these cells resulted in a decrease of the rate of oviposition and of shell growth. In this respect it is relevant to mention that an endogenous circadian rhythm in the oviposition of Lymnaea stagnalis, showing a peak during the night, is apparent (Van der Steen, 1970), whereas diurnal variations in the shell growth occur in many molluscan species (Pannella and MacClintock, 1968). It seems worthwhile to search for other body functions showing a diurnal rhythmicity to elucidate the function of the CDC. In this regard it is worth mentioning that during the present studies a distinct diurnal rhythm was observed in the copulation activity in Lymnaea stagnalis.

The rate of formation and of storage of the neurosecretory material of the CDC varies gradually during the 24 hr period. On the other hand, the release activity shows a sudden rise. This holds, although to a lesser degree, also for the LGC. This rise coincides with sunset, suggesting that release activity is effected by the change in light intensity. A relation with light does not seem unlikely, since light plays an important role in the regulation of diurnal rhythms (cf., Aschoff, 1965), as has been demonstrated for the rhythmical electrical (Lickey, 1968) and secretory (Berstein, 1967) activity of the particular neuron of Aplysia. In Lymnaea stagnalis further experimental evidence is needed to define the relationship between the secretory activity in the CDC and LGC, and light.

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