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Acid Phosphatase Localization in Neurons of *Bulla gouldiana* (Gastropoda: Opisthobranchia) *

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Summary. The organization of the ganglia and the ultrastructure of the neurons of *Bulla gouldiana* are similar to those described for other molluscs. Acid phosphatase positive reactions were found in the large pigmented granules, small dense bodies, multivesicular bodies, and Golgi lamellae and associated vesicles. The small dense bodies and multivesicular bodies may be stages in the formation of the larger pigmented granules which are interpreted as lysosomes. Comparison is made between the pigmented granules in *Bulla* and the lipofuscin bodies of vertebrate neurons. The possible involvement of these pigmented granules in the hyperpolarization of *Bulla* and *Aplysia* neurons to light is discussed.

Key words: Ganglia — Neurons — Pigmented granules — Acid phosphatase — Lysosomes.

Introduction

Gastropod neurons are well known for their large size and bright pigmentation which is due to the presence of large cytoplasmic granules. Reports on the anatomy of various ganglia and the ultrastructure of individual neurons in several gastropods include descriptions of the formation and accumulation of their pigmented granules (Chou, 1957a, 1957b; Chou and Meek, 1958; Bullock, 1961; Chalazonitis, 1961; Rosenbluth, 1963; Amoroso *et al.*, 1964; Bullock and Horridge, 1965; Arvanitaki and Chalazonitis, 1966; Chalazonitis *et al.*, 1966; Coggeshall, 1967; Simpson *et al.*, 1963). In *Helix aspersa*, *Planorbis trivolvis*, and *Tritonia diomedia* structures in neuron somata, called "lipochondria", "complex bodies", and "mixed lipid droplets", which resemble the pigmented granules in *Bulla*, have been positively identified as lysosomes (Lane, 1963, 1966; Meek and Lane, 1964; Adzhimolaev *et al.*, 1972).

Our investigations of *Bulla gouldiana*, a primitive cephalaspidean, reveal that the ganglia of this organism are bright red due to the accumulation of pigmented granules in the cytoplasm of most nerve cells. This report describes the ultrastructure and formation of these pigmented granules and the localization of acid phosphatase activity, thus identifying these structures as lysosomes of the residual

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body variety. The comparison of the pigmented lysosomes of *Bulla* with lipofuscin bodies that accumulate with age in vertebrate nervous tissue and the possible secondary role of lysosomes as primitive photoreceptors in some molluscan neurons are briefly discussed.

Materials and Methods

Bulla gouldiana were collected from mud-flat areas in Ventura County, California, and kept in the laboratory in running sea water aquaria. The population was maintained on freshly collected *Ulva* or lettuce.

The ganglia were removed from the animal, freed from most of their connective tissue capsules and fixed for 1–2 hours in 3% glutaraldehyde buffered in 0.1 M Sodium Cacodylate buffer overnight and then embedded in 8% agar. The resulting tissue blocks were cut in 50 μm sections on a Sorval tissue chopper, and the sections were then incubated for acid phosphatase, pH 5.0, at 37° C for periods of 15 and 30 minutes. The method of Gomori (1952), modified by Barka and Anderson (1962), was used for preparation of the incubation medium and β-glycerophosphate was used as substrate. After incubation, the tissue was washed in 0.1 M Sodium Cacodylate buffer, pH 7.0, for two hours and post-fixed in 2% OsO_4 for one hour. The dehydrated tissue was embedded in Araldite. Thin sections were cut on a Porter-Blum MT-2B ultramicrotome, left unstained, and examined by electron microscopy at 60 KV accelerating voltage. Control sections were incubated in media containing no substrate and in media made 0.01 M with respect to NaF.

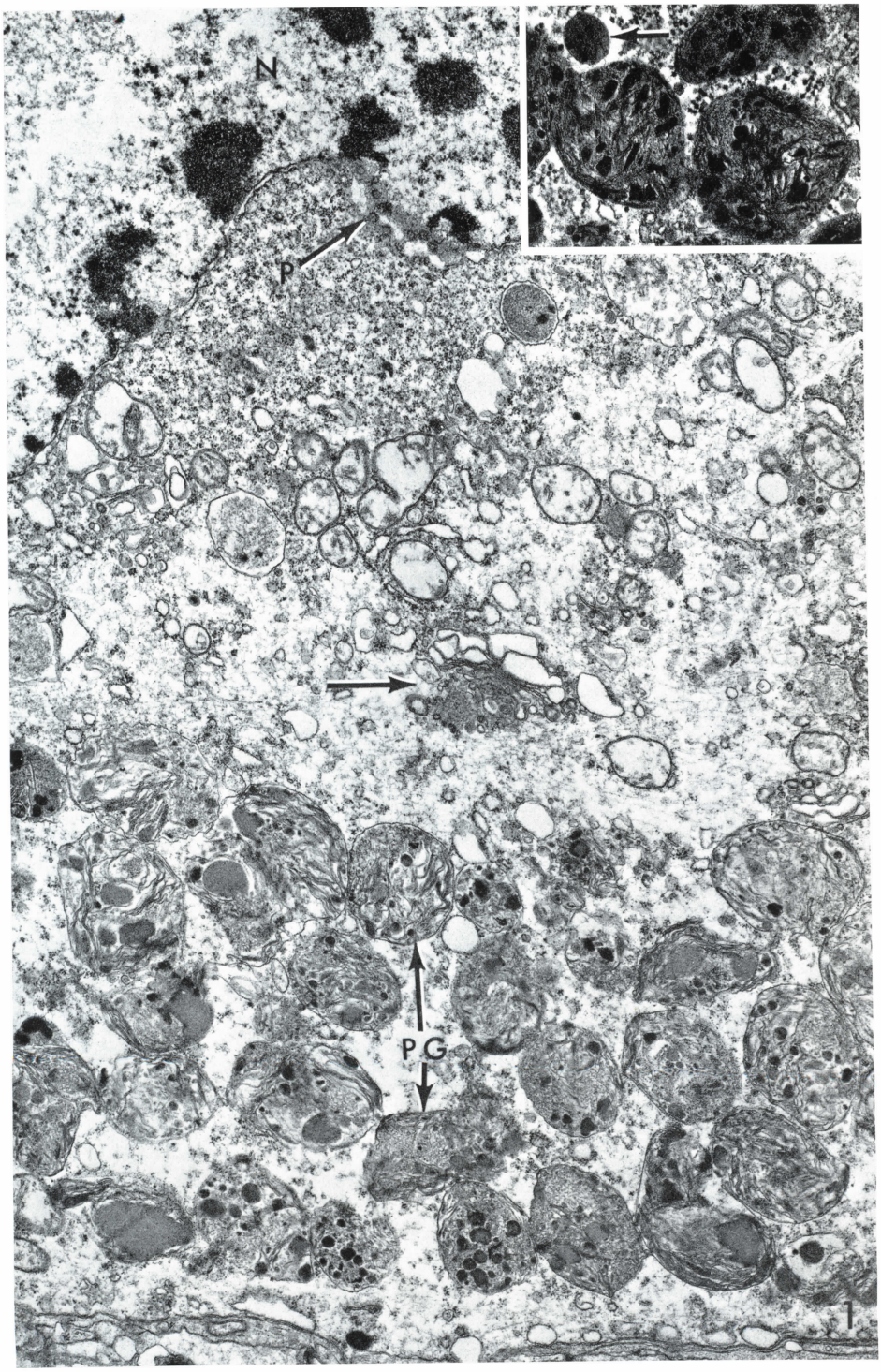
Some ganglia, examined for purely ultrastructural characterization (Figs. 1–5), were fixed in either 3% glutaraldehyde in 0.1 M phosphate buffer or in Karnovsky's fixative (Karnovsky, 1965) at pH 7.4. Whole ganglia were washed for two hours in the buffer, post-fixed in 2% OsO_4, stained *en bloc* overnight in 0.5% uranyl acetate in distilled water, dehydrated in a graded ethanol series, embedded in Araldite and sectioned as were the incubated tissues. Sections were stained with Reynold's lead citrate for 10 minutes and examined in a Siemens Elmiskop IA or a Phillips EM 300.

Results

General Morphology. The cerebral, parietal, pedal, pleural, buccal, supra- and subintestinal, and visceral ganglia were examined. All have the same general structure. In adult *Bulla* (shell length approximately 5 cm) neuron cell bodies range from 10–400 μm in diameter. The nuclei are large, lobate and contain many nucleoli. The perinuclear cytoplasm contains a high concentration of both free and bound ribosomes, smooth and rough endoplasmic reticulum, Golgi complexes, mitochondria, and numerous small vesicles of the plain and coated variety (Fig. 1).

The pigmented granules responsible for the bright red color of *Bulla* neurons are approximately 1–4 μm in diameter (Fig. 1) and occur primarily around the periphery of the cell body and in the axon hillock region. Electron micrographs

Fig. 1. Low power micrograph of a visceral ganglion neuron. Note lobated nucleus (*N*), nuclear pores (*P*), high concentration of rough endoplasmic reticulum and free ribosomes in perinuclear region, grouping of organelles (arrow) in cytoplasm adjacent to perinuclear region, and pigmented granules (*PG*) in peripheral cytoplasm. × 14000. Inset. Low power electron micrograph of pigmented granules in supra-intestinal ganglion neuron. Note dense lipid-like inclusions and membranous lamellae within granules, glycogen granules and small dense bodies (arrow) around pigmented granules. × 12500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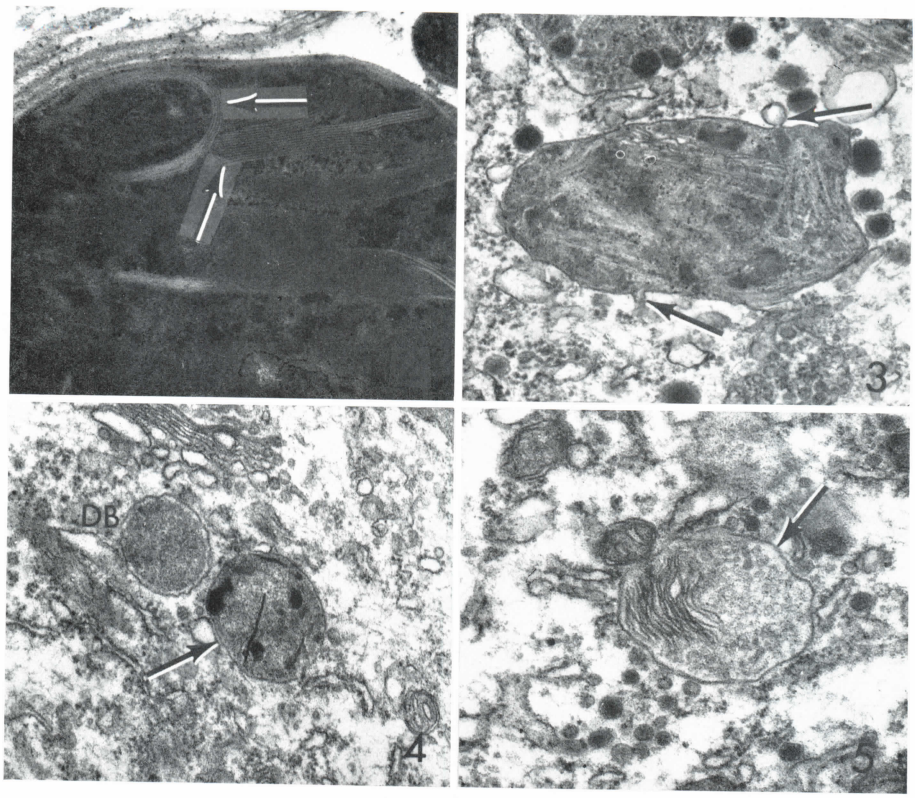


Fig. 2. Electron micrograph showing substructure of pigmented granule. Note typical lamellar arrangement of membranes (arrows) within granules and peculiar negative staining-like effect. × 33750

Fig. 3. Several small vesicles apparently fusing (arrows) with limiting membrane of neuronal pigmented granule. Visceral ganglion. × 33000

Fig. 4. Small dense bodies in perinuclear cytoplasm of cerebral ganglion neuron. Note entirely granular dense body (*DB*) and one containing lipid-like inclusions and lamellae (arrow). × 25000

Fig. 5. Multivesicular body (arrow) in cerebral ganglion neuron. Note membrane lamellae, small vesicles, and granular material. × 45000

reveal these pigmented granules to be bounded by a single membrane and to contain dense lipoid inclusions, granular material in varying amounts, and numerous lamellae (Figs. 1, 1-inset, 2). At higher magnification small vesicles are occasionally seen that appear to be fusing with the outer membrane of the pigmented granules (Fig. 3). Numerous glycogen granules also occur in the areas of pigmented granule accumulation (Fig. 1-inset).

Within the neuronal cytoplasm probable stages in the formation of the pigmented granules can be observed. In the perinuclear region dense bodies ranging in size from 0.5–1.0 μm are apparent which can be entirely granular or may contain single or concentric lamellae in conjunction with granular material

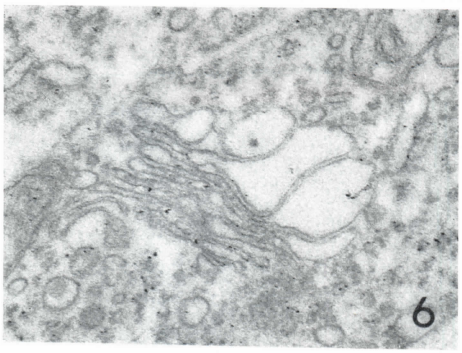
(Fig. 4). These dense bodies are seen near the cell periphery in close proximity to the larger pigmented granules (Fig. 1-inset). Structural similarities between the dense bodies containing lamellae, granular material and dense lipoid inclusions, and the larger pigmented granules are apparent. Multivesicular bodies are also numerous (Fig. 5); some contain vesicles and lamellae while others include dense lipoid or granular material. These two organelles may be intermediate structures in the formation of the large pigmented granules.

Enzyme Localization. Control sections incubated with no substrate and with NaF as an inhibitor showed no enzyme localization in both the 15 and 30 minute treated sections (Figs. 6–8). Since all ganglia showed similar results only micrographs for the buccal ganglia are shown. Fifteen minute incubation periods provided the best acid phosphatase localization. Marked acid phosphatase activity was seen in the large pigmented granules (Fig. 9) around the lamellar inclusions (Fig. 10). These results identify the pigmented granules as lysosomes. Within the perinuclear and central cytoplasmic areas, dense bodies and multivesicular bodies were strongly acid phosphatase positive (Figs. 11, 12). In addition, several of the Golgi saccules contained the lead precipitate (Fig. 13) as did large vesicles presumably arising from the Golgi apparatus (Fig. 14).

Discussion

Acid phosphatase reactions in *Bulla* are comparable to those in *Helix*, *Planorbis* and *Tritonia* (Adzhimolaev *et al.*, 1972; Lane, 1963, 1966; Meek and Lane, 1964). In *Helix* acid phosphatase activity was shown to be present in the "lipochondria" (Chalazonitis, 1964) and Golgi complex including both the Golgi vesicles and lamellae (Meek and Lane, 1964). In *Planorbis* acid phosphatase positive reactions were restricted to two or three of the Golgi saccules in the fenestrated area as well as in the lipochondria-like dense bodies (Lane, 1966). In the giant nerve cells of *Tritonia* material in the "complex bodies", similar to the pigmented granules in *Bulla*, exhibited a positive acid phosphatase reaction (Adzhimolaev *et al.*, 1972). In *Bulla* the results showed several Golgi saccules, multivesicular bodies, smaller dense bodies, and large pigmented granules to be acid phosphatase positive. The pigmented granules in *Bulla gouldiana* can thus be identified as lysosomes. In some sections smaller vesicles were seen fusing with the large pigmented granules; these could represent primary lysosomes in the process of delivering their contents to the larger secondary lysosomes (de Duve and Wattiaux, 1966). The lysosomes in *Bulla* seem mainly to arise as a result of autophagic events and the presence in the cytoplasm of acid phosphatase positive vesicles that contain what resemble degenerate cellular organelles supports this conclusion (Fig. 12).

Preliminary light microscopic staining procedures and fluorescence microscopy (Robles and Fisher, in preparation) have further classified the pigmented granules in *Bulla gouldiana* as lipofuscin bodies, or lysosomes of the residual body variety, which are similar in structure and staining properties to the lipofuscin granules that accumulate with age in vertebrate neurons. Pearse (1972) states that lipofuscin pigments are usually yellow to brown but may also be colored by dissolved carotenoids. In *Bulla* the observation of a dark red pigment in the granules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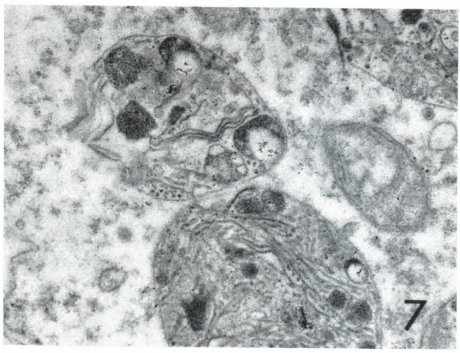
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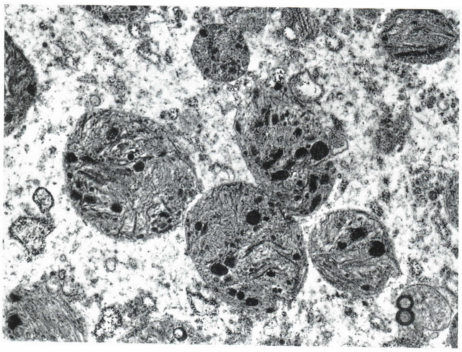
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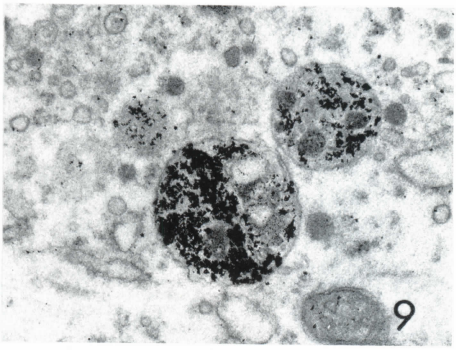
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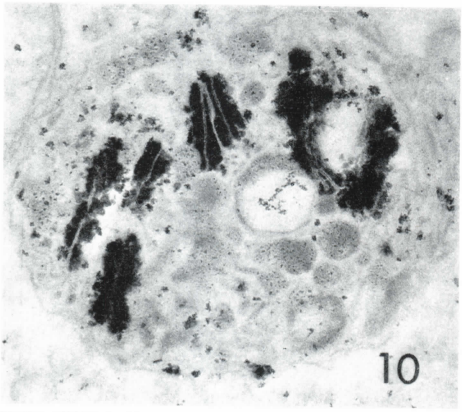
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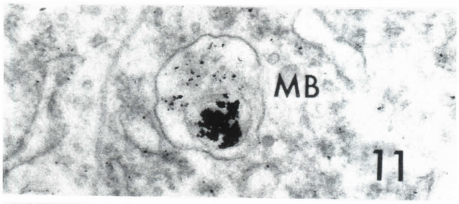
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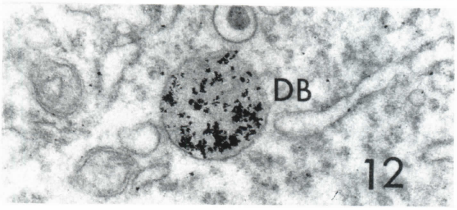
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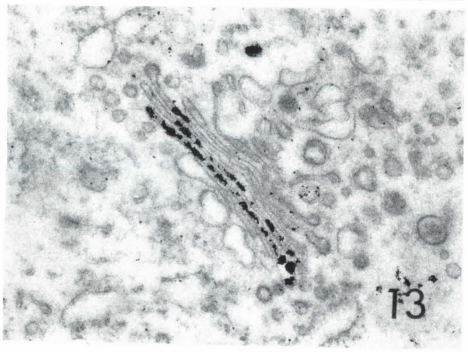
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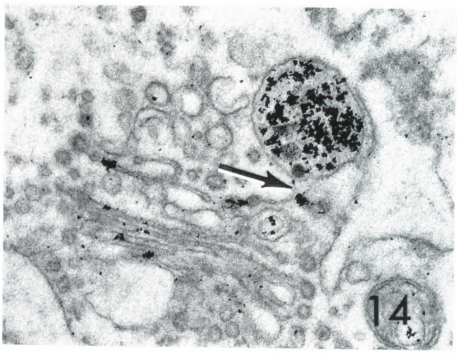
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suggests the presence of carotenoids. Spectrophotometric studies of extracted pigment confirm the presence of carotenoids in *Bulla* ganglia (Robles and Fisher, in preparation).

Another interesting finding in molluscan neurons is the possible involvement of the lysosomes, or pigmented granules, in the light sensitive response observed in some molluscan preparations. Frazier *et al.* (1967) reported hyperpolarization of several identified neurons in the abdominal ganglion of *Aplysia californica* in the presence of light. Later Brown and Brown (1972, 1973) studied the giant R2 cell in abdominal ganglia of *Aplysia* and showed that illumination of R2 evokes a membrane hyperpolarization which is associated with increased K^+ conductance. An action spectrum showed that the light response was best produced at 490 nm which corresponded to the absorption maximum of the neuron. Injection of Ca^{++} into R2 also evoked a hyperpolarization which was attributed to an increase in K^+ conductance. Brown and Brown (1973) concluded that the mechanism for hyperpolarization by light may involve the pigment which, when illuminated, causes a release of Ca^{++} and a concomitant increase in K^+ conductance.

Henkart (1973) fixed R2 cells in the light and dark and noted a change in their appearance. "Lipochondria" fixed in the dark appeared granular while those incubated in the light contained concentric lamellae. Also studies in which Sr^{++} was substituted for Ca^{++} in the incubating medium resulted in an altered appearance of the "lipochondria". Henkart suggests that the "lipochondria" are capable of binding divalent cations, and thus acting as the internal source of Ca^{++} during the light response.

Preliminary results in *Bulla* (Robles and Fisher, in preparation) have shown the pigmented neurons to be light sensitive. During intracellular recordings, a slow hyperpolarizing response of 2–3 mv was observed when the ganglion was illuminated with white light. Experiments are in progress to determine if the red pigment in the neurons is involved in this response.

Fig. 6. Acid phosphatase control, no substrate, incubated 15 minutes. Buccal ganglion. Unstained section. No acid phosphatase reaction product in organelles. × 30000

Fig. 7. Acid phosphatase control, no substrate, incubated 15 minutes. Buccal ganglion. Unstained section. No acid phosphatase reaction product in large pigmented granules. × 30000

Fig. 8. Acid phosphatase control, incubated 15 minutes, sodium fluoride present in medium. Buccal ganglion. Unstained section. No acid phosphatase reaction product in pigmented granules. × 30000

Fig. 9. Group of pigmented granules showing high acid phosphatase activity. Buccal ganglion. Incubated 15 minutes. Unstained section. × 30000

Fig. 10. Acid phosphatase localization product around lamellar inclusions of pigmented granules. Buccal ganglion. Incubated 15 minutes. Unstained section. × 99000

Fig. 11. Acid phosphatase localization product in multivesicular body (*MB*). Buccal ganglion. Incubated 15 minutes. Unstained section. × 25000

Fig. 12. Acid phosphatase localization product in dense body (*DB*). Buccal ganglion. Incubated 15 minutes. Unstained section. × 25000

Fig. 13. Acid phosphatase precipitate in Golgi apparatus. Buccal ganglion. Incubated 15 minutes. Unstained section. × 35000

Fig. 14. Positive acid phosphatase reaction in dense body (*DB*) and Golgi apparatus. Note that dense body appears to arise from Golgi lamellae (arrow). Buccal ganglion. Incubated 15 minutes. Unstained section. × 35000

In *Bulla gouldiana* and a few other gastropods, acid phosphatase reactions characterize the pigmented granules as lysosomes which suggests a digestive function. However, the possibility remains that the pigmented granules may also mediate the light sensitive response, and thus may be acting as primitive photoreceptors.

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