Research Article

Ultrastructural Comparison of Processing of Protein and Pigment in the Ink Gland of Four Species of Sea Hares

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The ink glands of four sea hare species (*Aplysia californica*, *A. parvula*, *A. juliana*, and *Dolabrifera dolabrifera*) were compared to determine where ink protein is synthesized, how it is incorporated into protein storage vesicles, and the degree of variation in the structure of the ink gland. Ink protein was synthesized in RER cells and stored in amber and white vesicles. Lack of competent RER cells in the ink gland of *D. dolabrifera* was correlated with the absence of ink protein. Ink protein had similar characteristics in all three *Aplysia* species but, again, it was absent in *D. dolabrifera*. Its uptake involved pinocytosis by protein vesicle cell membranes. Granulate cells showed little variation in structure among the four species, the opposite was the case for RER cells. The conversion of the red algal pigment, phycoerythrin, to phycoerythrobilin (PEB) occurs in the digestive gland but the change of PEB to aplysioviolin (APV), the form of pigment released by the ink gland, occurs in the ink gland itself by both granulate cells and pigment vesicles. The literature describes five types of vesicles based upon color and contents in the ink gland of these four species. We report only three types of vesicle: colored (purple), protein (white and amber), and transparent (includes clear vesicles).

1. Introduction

All species of sea hares (Gastropoda: Euopisthobranchia: Anaspidea) have an ink gland and release white ink, purple ink, both colors together, or none at all [1–4]. Ink of the best-studied-ink-producing sea hare species, *Aplysia californica* Cooper, 1863, consists largely of two components; 65% is phycoerythrobilin (PEB), the red algal photosynthetic pigment, r-phycoerythrin, minus its low molecular mass protein [2, 5–9] while 35% is high molecular weight protein. Kamio et al. [10] report, however, that aplysioviolin (APV, the methylated form of PEB) and not PEB is the abundant form of purple pigment in ink. All previous studies that characterized sea hare pigments used techniques that could not distinguish between PEB and APV.

The second component of ink, the high molecular mass ink protein, is synthesized by the sea hare itself [3, 9]. This ink protein has been isolated and sequenced for several sea hares. In *Aplysia californica* it is called escapin due to its antipredator properties which have been demonstrated against sea anemones, spiny lobsters, and fish [2, 11–13]. In

A. kurodai it is named aplysianin-P [6, 7], in *A. juliana*, julianin-S, [14], in *A. dactylomela*, dactylomelin-P [15, 16], in *Bursatella leachii*, bursatellanin-P [17], and, in *A. punctata*, *Aplysia punctata* ink toxin [18]. All of these ink proteins have molecular weights of approximately 60 to 70 kDa, are bioactive, and most are known to have both antimicrobial and antitumor activity [2, 6, 7, 14–17].

Ultrastructural comparison of both the ink glands [3] and digestive glands [19] of sea hares that produce ink of several colors (purple ink in *Aplysia californica*; white and purple ink in *A. parvula* Guilding in Morch, 1863; only white ink in *A. juliana* Quoy and Gaimard, 1832; or no ink at all in *Dolabrifera dolabrifera,* Cuvier, 1817) should help elucidate (1) the pathways and cell types involved with the formation of the colored component of ink; (2) the site of synthesis, uptake, and storage of the protein constituent; (3) whether these processes are the same in different sea hares; (4) whether the pigment and protein components of ink always are stored in the same vesicle type; (5) why different sea hares release ink of different color; (6) why one sea hare species releases no

ink, neither its pigment nor protein component; and (7) the proximate reason for color variation in the ink produced by different sea hare species.

2. Materials and Methods

Four sea hare species were collected from several different geographical locations (described in [3, 9, 19, 20]). Adults of *Aplysia juliana* (150–200 g wet mass) from Noto Peninsula, Ishikawa Prefecture, Japan, had only white vesicles as they were feeding on their preferred diet of green algae, *Ulva* and *Enteromorpha* spp. Adults of *A. parvula* (1-2 g wet mass) were collected from intertidal areas near the University of Guam Marine Laboratory. Seven adults of *D. dolabrifera* (4 g wet mass) were collected from the bottom of several outdoor tanks at the above Marine Laboratory. Five specimens of *A. californica* (100–200 g wet mass) were obtained from the National Center for Research Resources *Aplysia* Facility, University of Miami, where they were fed the red alga, *Gracilaria tikvahiae*. Individuals (2–5 of each species) were anesthetized (isotonic magnesium chloride) and their ink glands removed and prepared for electron microscopy. The tissue was fixed in 2.5% glutaraldehyde in half-strength Millonig's phosphate buffer, pH 7.3, for 2 h at room temperature, postfixed in 1% OsO_4 for 1.5 h, dehydrated in an ethanol series, and embedded in LR White resin [20]. Individual amber and clear vesicles (3 of each vesicle type) of *A. californica* and the entire ink gland of *A. juliana* were isolated and fixed to prevent protein hydration. For this latter fixation 1.5% formaldehyde was added to the above fixative and the tissue then processed in a similar fashion. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed with a Philips 300 electron microscope at 60 kV or a JEOL 1400 at 80 kV. Center-to-center distances between adjacent cells of the protein lattice were measured directly from negatives using an ocular micrometer. Various other measurements (caveolae per cell membrane length, tubule dimensions, and membrane thickness) were determined by digitizing photomicrographic negatives of stained thin sections and then using Image J 1.40 for Macintosh. Semithin sections (1 μm) stained with Toluidine Blue provided cell characteristics for various cell types.

3. Results

The general ultrastructural architecture of the ink gland and the cell and vesicle types found in this gland are described below first for *Aplysia californica* and then for the other species of sea hares.

In *A. californica* the RER and granulate cell types and vesicles make up the ink gland [9]. The RER cell type is characterized by an extensive rough endoplasmic reticulum (rer) filled with filamentous material of low electron density and the presence of a nucleolus (Table 1). The granulate cell type is characterized by several dense pigment storage granules, sieve areas (periodic modification of the cell membrane into complex pores, Fig. 11, in [9]), and the lack of a nucleolus (Table 1).

Prince et al. [9] found, however, that the literature on the nomenclature of the vesicles (secretory cells) in the ink gland was unclear. They proposed naming the large secretory cells surrounded by muscle, ink-release vesicles, and vesicle cell prior to its enclosure by muscle. A further modification in terminology of ink-release vesicles is appropriate based upon the current study. We propose the terms (1) vesicle, for mature, large secretory cells surrounded by muscle, regardless of contents, (2) vesicle initial, for the stage not yet fully enclosed by muscle, and (3) vesicle cell, for young vesicles totally devoid of an external muscle layer (Figure 1). Vesicle could be further subdivided into those that release pigment (purple vesicles), protein (amber and white vesicles) and those that release nothing and the contents are electron-lucent (clear and transparent vesicles; Table 1).

3.1. Aplysia californica (Releases Purple Ink). The vesicles that release purple ink are the only vesicle type described ultrastructurally. Amber and clear vesicles were imaged only by light microscopy [9].

3.1.1. Amber Vesicles. Individual amber vesicles of *A. californica* prepared with only the glutaraldehyde fixative contained various-sized vacuoles filled with hydrated protein appearing as medium electron dense filamentous to particulate material (Figures 2(a), 2(d), and 2(e)). When prepared with the glutaraldehyde/formaldehyde fixative, membrane bound, crystalline protein granules were observed within the cytoplasmic zone adjacent to the muscle wall (Figures 2(b) and 2(c)); however, large vacuoles of hydrated protein remained. The center-to-center distance between adjacent cells within the lattice of the protein crystal was 37 ± 6 nm (mean distance $\pm$ SD, $n = 14$; Table 1). These small protein vacuoles appeared to fuse with the larger vacuoles that contained hydrated protein (Figure 2(e)), the membrane of the former becoming part of the large vacuole membrane resulting in its increase in size.

Vesicle cells were characterized by (1) a large size, compared to adjacent cells; (2) a large prominent nucleus with abundant heterochromatin; (3) a dense, organelle filled cytoplasm; (4) cisternae of rough endoplasmic reticulum closely associated with the cell membrane; (5) invaginations of the vesicle cell membrane itself to form sack-like internal projections, these filled with low density material; and (6) the lack of any muscle outside of the cell membrane (Figures 1(a), 2(a), 2(f), and 3(a)).

Ducts of amber vesicles appeared to have few microvilli on the side facing the duct lumen (Figure 3(b)). The lumen itself was filled with medium density material (probably protein).

3.1.2. Clear Vesicles. Each clear vesicle had a layer of vacuolated cytoplasm pressed against the vesicle membrane and muscle wall (Figure 4(a)). The cytoplasmic layer was separated from an electron-lucent interior by a membrane often covered by an electron dense, amorphous layer. Vacuoles with a membrane thickness of approximately 8 nm ($n = 2$) were observed in the cytoplasmic area and contained stacks of sheets (Figures 4(b) and 4(c)). Each sheet (total

TABLE 1: Characteristics of the ink gland of four species of sea hare.

	Sea hare species			
	A. californica	*A. juliana*	*A. parvula*	*D. dolabrifera*
RER cells				
Abundant rer	+	+	+	−
Condensed rer	−	+	−	−
Microtubule in vacuoles	−	−	+	−
Number of caveolae[1]	0.4 ± 0.2[2]	0.2 ± 0.3[2]	3.3 ± 0.7[2]	2.3 ± 0.4[2]
Caveolar shape	Balloon	Balloon	Balloon	Long and Branched
Nucleolus	+	+	+	−
Granulate cells				
Nucleolus	−	+	+	−
Sieve areas	+	+	+	+
Granules	Diet dependent	+	+	−
RER and granulate cell				
Abundance	Many	Many	Many	Few
Spacing in gland	Close	Close	Close	Dispersed
Vesicle type				
Purple	Pigment	Pigment	Pigment	−
Amber[4]	Protein	−	−	−
Clear[5]	Electron-lucent with sheets	−	−	−
White[4]	−	Protein	Protein	−
Transparent[5]	−	−	−	Electron-lucent with tubes
Protein in vesicles				
Lattice dimensions	37 ± 6 nm[2]	46 ± 10 nm[2]	36 ± 11 nm[2]	15 ± 2 nm[2,3]

(1) = number/length of cell membranes in μm; (2) = mean ± SD; (3) = in cells beneath epidermis; (4) = amber and white vesicles now termed protein vesicles; (5) = clear vesicles now termed transparent vesicles.

thickness of 4.3 nm ± 1.0 nm; mean thickness ±SD, $n = 32$) was composed of two electron dense layers separated by an electron transparent zone.

3.2. Aplysia juliana (Releases White Ink). *Aplysia juliana* releases white ink when feeding on its preferred diet of green algae. When restricted to a diet of red algae (*Porphyra* sp.), however, it produces purple vesicles in addition to white vesicles [1, 3].

RER cells of *Aplysia juliana* were characterized by extensive rough endoplasmic reticulum (rer) whose cisternae were seldom expanded (Figure 5(a)). Expanded areas of rer cisternae contained material of medium electron density similar to that found in rer cisternae of RER cells of *A. californica* (Figures 5(a)–5(c) compare to Figs 6C, 8A, in [9]). Rer formed circular arrays or Golgi-like stacks, the ends of which were expanded and contained material of medium electron density (Figures 5(b) and 5(c)). RER cells had 0.2 ± 0.3 (mean ± SD, $n = 15$; Table 1) caveolae per length of cell membrane and their nuclei had nucleoli.

Granulate cells had sieve areas, a nucleolus, and numerous dense granules (Figure 5(d)). Little intercellular space occurred between cells and vesicles of the ink gland.

3.2.1. White Vesicles. Contents of mature white vesicles were stratified into two to three zones (Figure 4(d)). The first,

adjacent to the muscle wall, contained cellular organelles, vacuoles, and numerous protein crystals. A membrane separated this first layer from the next two that were characterized by decreasing densities of material. Protein crystals were not enclosed in a membrane and had a center-to-center distance of 46 ± 10 nm (mean distance ± SD; $n = 10$; Table 1; Figure 4(f)). The vesicle membrane against the enclosing muscle layer was frequently highly invaginated, forming a system of closely associated, blind, canal-like structures (Figure 4(e)). These canals were filled with medium density material similar to that within distended rer cisternae of RER cells.

3.3. Aplysia parvula (Releases White and Purple Ink). *Aplysia parvula* releases both white and purple ink at the same time from individual white and purple vesicles in its ink gland [2]. RER cells of *A. parvula* contained dense granules, as seen with the light microscope, and thus appeared similar to granulate cells at a similar magnification. Transmission electron microscopy resolved these granules as vacuoles containing numerous short, microtubule-like inclusions. These inclusions collectively gave the vacuoles the dense appearance of a granule with the light microscope (Figures 5(e) and 5(f)). The diameter of the inclusions was 32.9 nm ± 4.2 (mean diameter ± SD, $n = 15$), wall thickness 9.43 nm ± 1.8 (mean thickness ± SD, $n = 10$), and electron transparent core
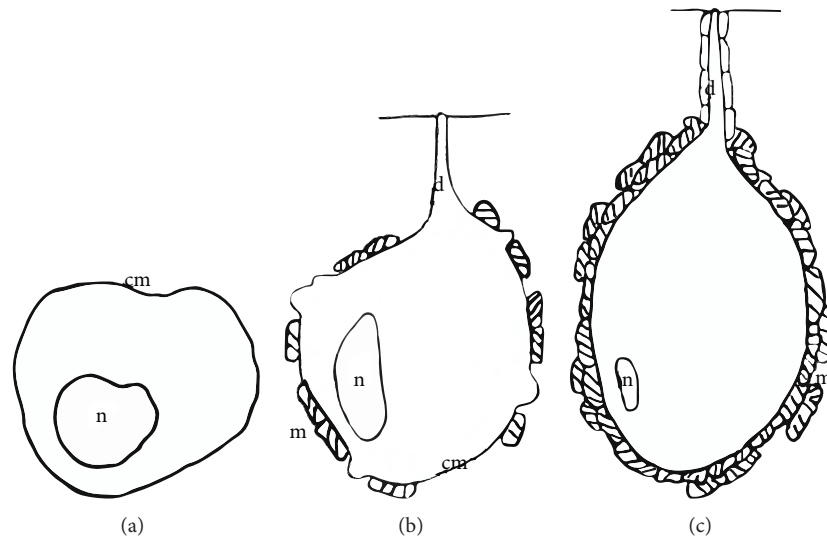
Figure 1: Stages in the development of a vesicle (not to scale). (a) Vesicle cell. (b) Vesicle initial. (c) Vesicle. cm, cell membrane; d, duct; m, muscle; n, nucleus.

approximately 14 nm. RER cells had 3.3 ± 0.7 (mean $\pm$ SD, $n = 20$) caveolae per length of cell membrane and their nuclei had nucleoli (Table 1).

Granulate cells (not shown) appeared similar to those in *A. juliana* (with sieve areas, pigment granules, little rer, and a nucleolus in the nucleus, Table 1). RER and granulate cells filled the ink glands of *A. parvula* leaving scant space between them and the vesicles, a similar situation to that of *A. juliana* and *A. californica*.

3.3.1. Purple Vesicles. Two types of vesicles occurred, periodically side-by-side. One contained pigment (purple vesicles) and the other protein (white vesicles, Figures 6(a) and 6(b)). Both types of vesicles contained a band of cytoplasm with various organelles adjacent to the vesicle membrane and the surrounding muscle wall. This cytoplasmic zone was separated from the interior contents by a membrane (Figures 6(a) and 6(b)). Contents of purple vesicles appeared as a mass of electron dense particles (Figure 6(a); see also Fig. 13 A-C in [9]). Vesicle cells (not shown) had an appearance similar to that described for *A. californica* above.

3.3.2. White Vesicles. Hydrated protein in white vesicles appeared as diffuse, thin strands of low to medium electron density dispersed among variously sized vacuoles (Figures 6(a) and 6(d), see also Figs 6C, 8A, in [9]), similar to the appearance of hydrated protein in amber vesicles of *A. californica*. Membrane-enclosed protein crystals were found in the cytoplasmic layer against the muscle wall. Since a double fixative was not used for *A. parvula* (as for *A. californica* amber and clear vesicles and the ink gland of *A. juliana*), the protein crystals were poorly preserved and dimensions (center-to-center distance of 36 ± 11 nm; mean distance $\pm$ SD; $n = 11$; Table 1) were difficult to ascertain. The cell membrane adjacent to the muscle layer was frequently invaginated to form elongate vase shaped structures that were

filled with filamentous material similar to that within the protein vesicle itself (Figures 6(c)–6(e)). This was similar to that seen in white vesicles of *A. juliana*.

3.4. Dolabrifera dolabrifera (Does Not Release Ink). The ink gland of *D. dolabrifera*, unlike those described above, consisted largely of muscle immersed in copious collagen and connective tissue, vesicles, and few RER and granulate cells. The RER cell type was characterized by numerous, elongate, branched caveolae, infrequent rer cisternae neither inflated nor filled with filamentous material of low electron density, and nuclei without nucleoli. Granulate cells lacked dense granules [3].

Membrane bound protein crystals with lattice dimension of 15 ± 2 nm (mean distance $\pm$ SD; $n = 11$) were found in elongate cells beneath the epidermis covering the ink gland but not in epidermal areas covering vesicles of this gland (Figure 7(f); Table 1). The protein crystals were frequently organized in a linear array in these cells.

A second cell type was found within or just beneath the epidermal layer of cells. Each of these cells had a prominent nucleus and six or more electron dense granules, each within a vacuole (Figure 7(g)). Each granule was separated from its membrane by an electron transparent space; the latter could be a fixation artifact. This cell type had all the hallmarks of pigment cells found below the epidermis of *A. californica* [21]. Pigment cells with their abundant purple pigment vacuoles are the principle source of the light, reddish-maroon skin color for mature *A. californica* where there are few pigmented epidermal cells [21].

3.4.1. Transparent Vesicles. Each transparent vesicle, like the clear vesicles of *A. californica* above, had a layer of vacuolated cytoplasm against the muscle wall and this was separated from an electron-lucent interior by a membrane against which an amorphous electron dense layer was deposited
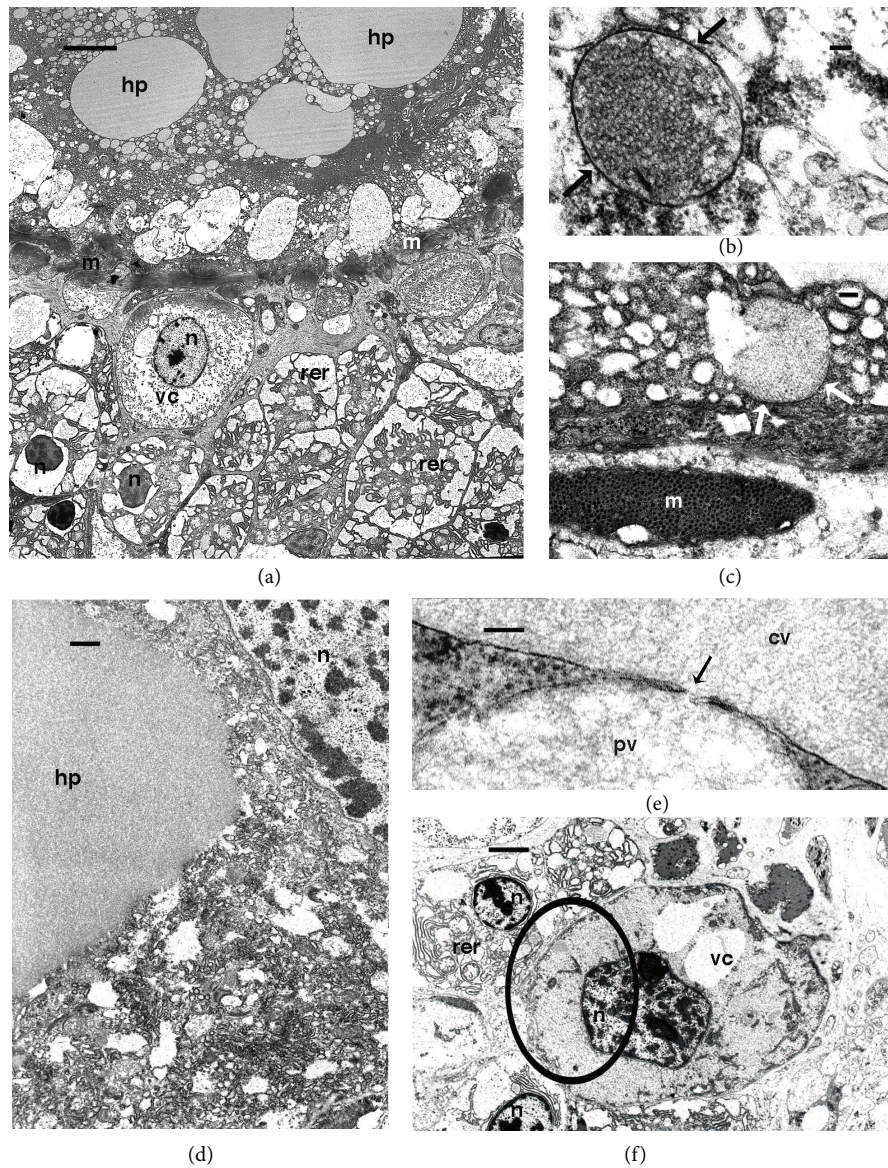
FIGURE 2: Amber vesicle of *Aplysia californica*. (a) Vesicle containing large vacuoles of hydrated protein. The amber vesicle is surrounded by cells rich in rough endoplasmic reticulum (RER cells) and is adjacent to a vesicle cell (see also (f)). ((b) and (c)) Protein contained within membrane bound vacuoles (arrows) in an amber vesicle. (d) Amber vesicle nucleus adjacent to the vesicle lumen which is filled with hydrated protein. (e) Fusion of protein vacuole membrane with central vacuole membrane (arrow) that fills the vesicle lumen. (f) Vesicle cell with large nucleus and dense cytoplasm. Circled area enlarged as Figure 3(a). cv, central vacuole; pv, protein vacuole; hp, hydrated protein; m, muscle; n, nucleus; rer, rough endoplasmic reticulum rich cell; vc, vesicle cell. Scale bars: (a) = 5 μm; (b) = 100 nm; (c) = 200 nm; (d) = 1 μm; (e) = 100 nm; (f) = 2 μm.

(Figures 7(a) and 7(b) compare to Fig. 4B, in [3]). No proteinaceous material was seen within the vesicles. Arrays of tubules (10.8 ± 10.2 nm; mean diameter ± SD, $n = 44$) were seen free within the vesicle interior (Figures 7(c)–7(e)). Cross-sectional views of these tubules (Figures 7(d) and 7(e)) showed a wall (thickness of 3.7 ± 0.7 nm; mean thickness ± SD, $n = 6$) surrounding a translucent core (4.7 ± 0.7 nm; mean diameter, SD, $n = 6$). The wall of the tubule was composed of 7 ($n = 3$) subunits, 3.4 ± 0.3 nm (mean ± SD, $n = 11$) in diameter (Figure 7(e)).

4. Discussion

4.1. Ink Gland Ultrastructure. The ink glands of four sea hare species can be readily distinguished at the ultrastructural level based upon an array of characteristics (Table 1). RER cells of the four species can be separated by the abundance of rer, the degree of expansion of rer cisternae, and the occurrence of a nucleolus. RER cells of *A. parvula* had aggregations of short microtubule-like rods that appeared with the light microscope as dense granules. But dense granules character-ize the granulate cell type at the same magnification. In the

(a)   (b)
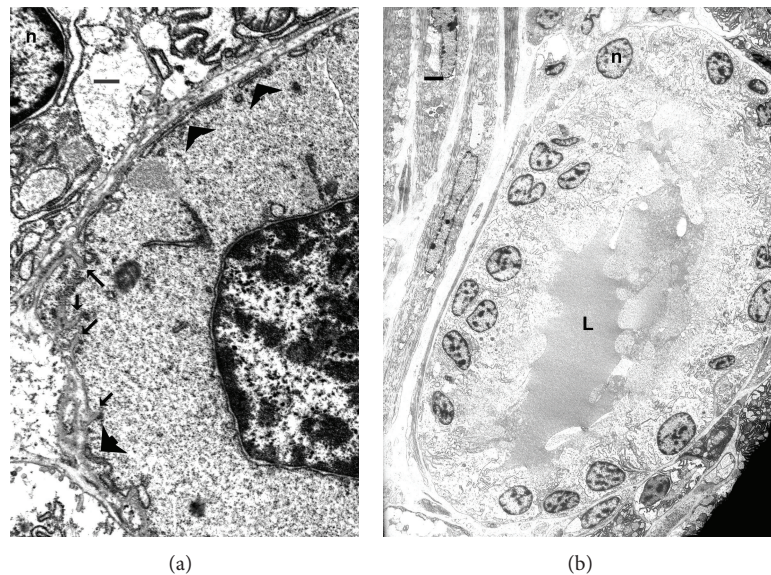
FIGURE 3: Vesicle cell and duct from amber vesicle of *Aplysia californica*. (a) Enlarged portion of vesicle cell from Figure 2(f) with sack-like invaginations (arrows) of the cell membrane which is also closely associated with rough endoplasmic reticulum cisternae (arrow heads). (b) Duct lumen filled with protein. L, lumen; n, nucleus. Scale bars: (a) = 0.5 μm; (b) = 2 μm.

granulate cell type, pigment aggregations provide this density, not aggregations of short rods (Fig. 8A, B, in [9]).

The number of caveolae per length of RER cell membrane and their shape also separated the sea hares (Table 1). *Aplysia parvula* had more caveolae per cell membrane than any other sea hare. Cells from *A. juliana* and *A. californica* had approximately the same number of caveolae per unit length of cell membrane but fewer caveolae than either *A. parvula* or *D. dolabrifera*. In addition, caveolae in *D. dolabrifera* consisted of long tubular, frequently branched structures [3] while in all the other sea hares they were balloon shaped. (For a review of caveolae, see [3].)

Sieve areas characterized granulate cells in all four species of sea hares. Otherwise they varied as to the presence of nucleoli (present only in *A. parvula* and *A. juliana*) and the presence of granules in the cell (absent only in *D. dolabrifera,* Table 1). One of the principal distinctions between the granulate cell type and rhogocyte cell type (for a review of these cell types, see [3]) is the presence of a nucleolus, generally, but not always, present in the rhogocyte but absent in the granulate cell type. Several additional traits, both functional and structural, distinguish the granulate cell type of *A. californica* and *D. dolabrifera* from rhogocytes [3]. Does the presence of a nucleolus in the granulate cell type in *A. parvula* and *A. juliana* suggest that they should be considered a rhogocyte rather than a granulate cell type? This will require further careful study of these two species of sea hare.

The ink gland of three species of sea hare, *A. californica*, *A. juliana*, and *A. parvula*, can be separated, furthermore, from those of *D. dolabrifera* based upon the spacing and frequency of vesicles, granulate, and RER cells. *Dolabrifera dolabrifera* has few if any of these and those that occur are separated by copious fibrous connective tissue [3] while little intercellular space occurred between these components in the ink glands of the other three species of sea hare.

4.2. Ink Color and Its Variation. *Dolabrifera dolabrifera* does not produce colored ink. This is not due to (1) the absence of appropriate digestive machinery, that is, rhodoplast cells, (2) the failure of rhodoplast cells to digest rhodoplasts, or (3) the absence of granulate cells and vesicles in the ink gland for pigment accumulation and storage [3, 19]. *Dolabrifera dolabrifera's* failure to form ink, therefore, is not due to the absence of an appropriate digestive or storage mechanism for pigment manipulation. Kamio et al. [22] found, however, that APV and not PEB was the predominant form of red pigment in ink and the ink gland; the reverse holds for the digestive gland. Perhaps PEB must be converted to APV in the digestive gland before it can be transported by the hemolymph and/or taken up by granulate cells and vesicles.

But pigment cells were found below the epidermis of the ink gland of *D. dolabrifera*. Pigment cells provide the light, reddish-maroon skin color for mature *A. californica* [21]. PEB predominates in *A. californica* except for the ink gland where APV is abundant [22]. Pigment cells in *D. dolabrifera,* ultrastructurally identical to those in *A. californica,* should have pigment in the form of PEB and not APV. In both *A. californica* and *D. dolabrifera* PEB must move from the digestive gland to pigment cells via the hemolymph and then is taken up by these cells.

The lack of pigment in vesicles and granulate cells of *D. dolabrifera* suggests that this species is not able to convert PEB to APV since ink pigment is in the form of APV [22]. The other three sea hare species do release colored ink. Their granulate cells and vesicles, therefore, are able to convert PEB to APV. The ink gland and not the digestive gland of *D. dolabrifera* is responsible for its lack of ink production.
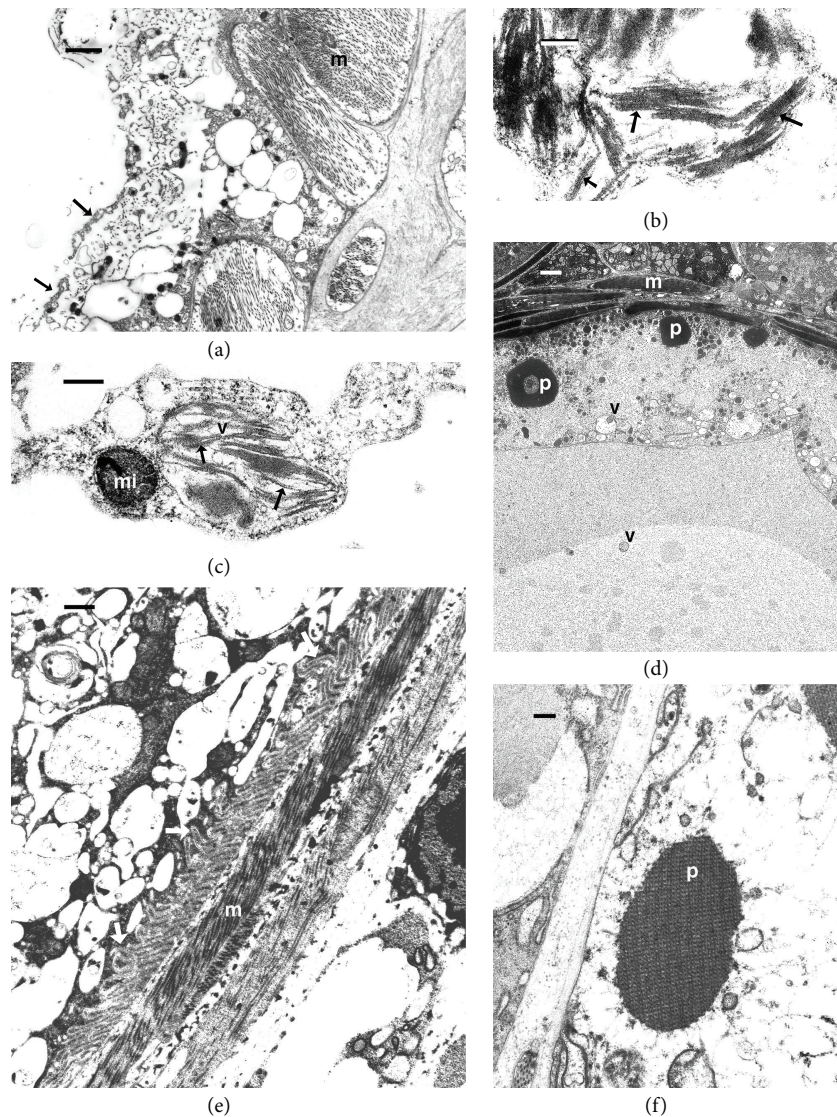
FIGURE 4: Clear vesicle of *Aplysia californica* ((a)–(c)) and white vesicle of *A. juliana* ((d)–(f)). (a) An electron-lucent lumen surrounded by a thin layer of cytoplasm bordered by muscle. The cytoplasm frequently had an electron dense material (arrows) against it. ((b) and (c)) Parallel arrays of sheets (arrows) contained within a cytoplasmic vacuole (c) within a clear vesicle. (d) Vesicle contents are frequently separated into three zones of decreasing density, the one closest to the muscle wall containing protein crystals. (e) Vesicle cell membrane highly infolded with the folds (white arrows) containing filamentous, low electron dense material. (f) Protein crystals are not enclosed by a membrane. m, muscle; mi, mitochondrion; p, crystalline protein; v, vacuole. Scale bars: (a) = 2 μm; ((b) and (c)) = 200 nm; (d) = 2 μm; (e) = 0.5 μm; (f) = 0.25 μm.

Variation in the color of released ink, white versus purple ink [1], is based upon diet preference (a white ink-producing sea hare, *A. juliana*, consumes green algae [3]) and not a difference in the structure of the ink gland. Individuals of *A. juliana* that are switched to a red algal diet also produce purple vesicles, along with the white ones that preexisted [3]. Indeed, individuals of *A. parvula* produce both white and purple ink [2] but Johnson suggests (unpublished data) that this is due to a ratio of nearly 1 : 1 between white and purple vesicles in its ink gland. *Aplysia californica* with a similar ratio of amber and purple vesicles could also produce ink of two colors but the number of purple vesicles on average ranges

between four and six times more than amber ones in this sea hare (see Figs 1B, 2C, D. in [9]). In both *A. parvula* and *A. californica* white or amber vesicles release their contents, which are then mixed as they move from the mantle cavity through the siphon [1], the amber color being overwhelmed by purple pigment.

4.3. Characterization, Site of Synthesis, Uptake, and Storage of Ink Protein

4.3.1. Characterization. The high molecular weight ink protein has been chemically characterized for *Aplysia californica*
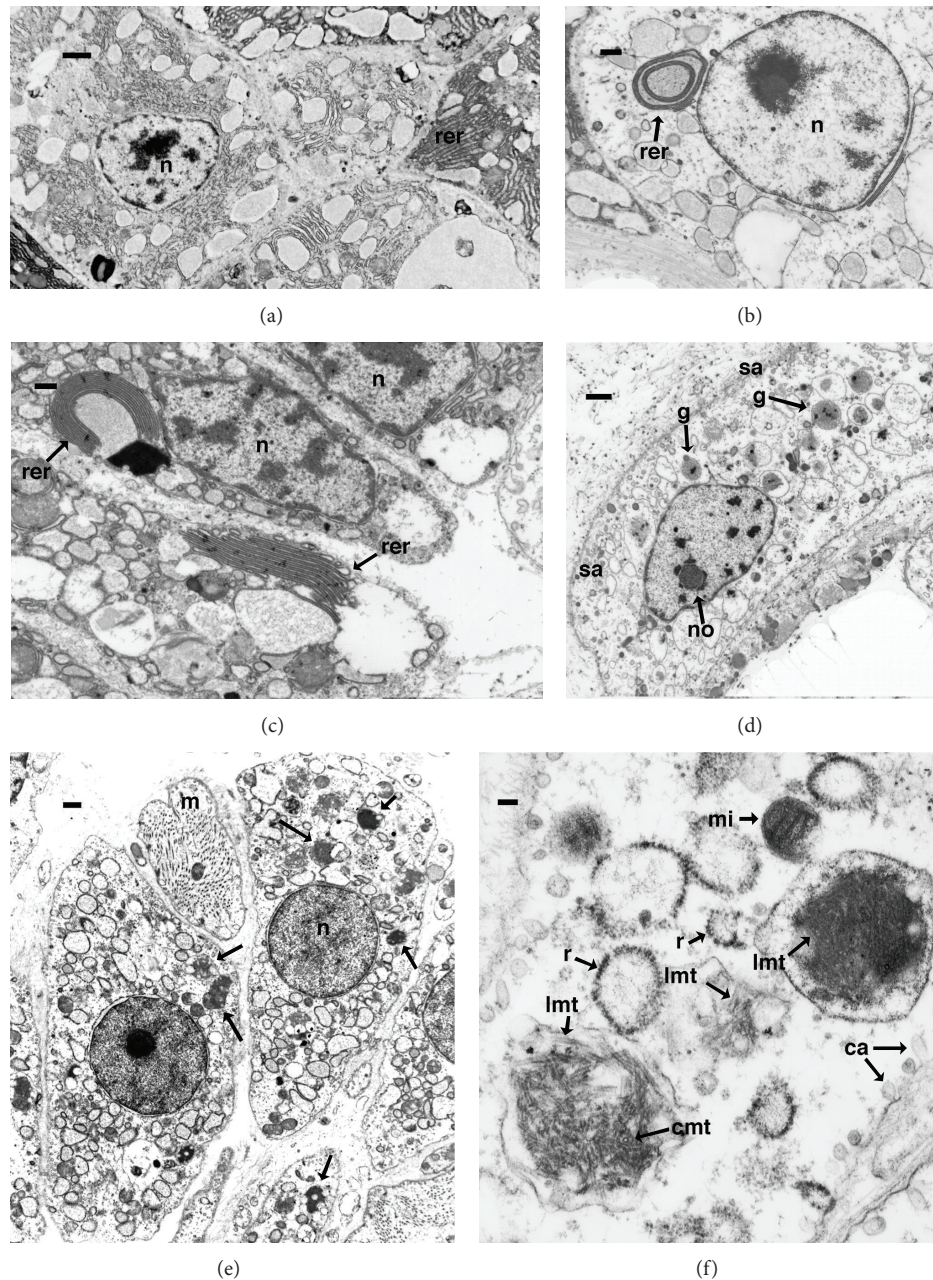
FIGURE 5: RER and granulate cells in the ink glands of *Aplysia juliana* ((a)–(d)) and *A. parvula* ((e) and (f)). (a) In RER cells the rer is closely appressed, only expanding into few vacuoles containing material of medium electron density or occurring as a whorl adjacent to the nucleus (b) or a Golgi-like stack of membranes with inflated tips (c). (d) A granulate cell has numerous dense granules, sieve areas, and a nucleolus. (e) RER cells have vacuoles containing dense material (arrows) appearing as dense granules with light microscopy (and similar in appearance to those in granulate cells). (f) These vacuoles contained aggregations of short, microtubule-like rods. ca, caveolae; Cmt, cross section of microtubule; g, dense granule; Lmt, longitudinal view of microtubule; m, muscle; mi, mitochondrion; n, nucleus; no, nucleolus; r, ribosome; rer, rough endoplasmic reticulum; sa, sieve area. Scale bars: (a)–(e) = 10 μm; (f) = 100 nm.

and *A. juliana* and is stored in amber and white vesicles, respectively (see Section 1 for protein analysis, below for vesicle storage types, and Table 1). The crystal dimension of ink protein in both types of vesicle was similar in concert with their similar molecular weights and mode of action (Table 1). The ink protein in the white vesicles of *A. parvula* has yet to be chemically analyzed but is probably similar in molecular weight and biological activity to that of *A. californica* and *A. juliana* since crystal dimensions were similar for all three species (Table 1). The protein in the ink gland of *D. dolabrifera* was different, however, from that in the other sea hares based upon its shorter lattice dimensions and its location in subdermal cells of the ink gland rather than in vesicles themselves (Table 1).
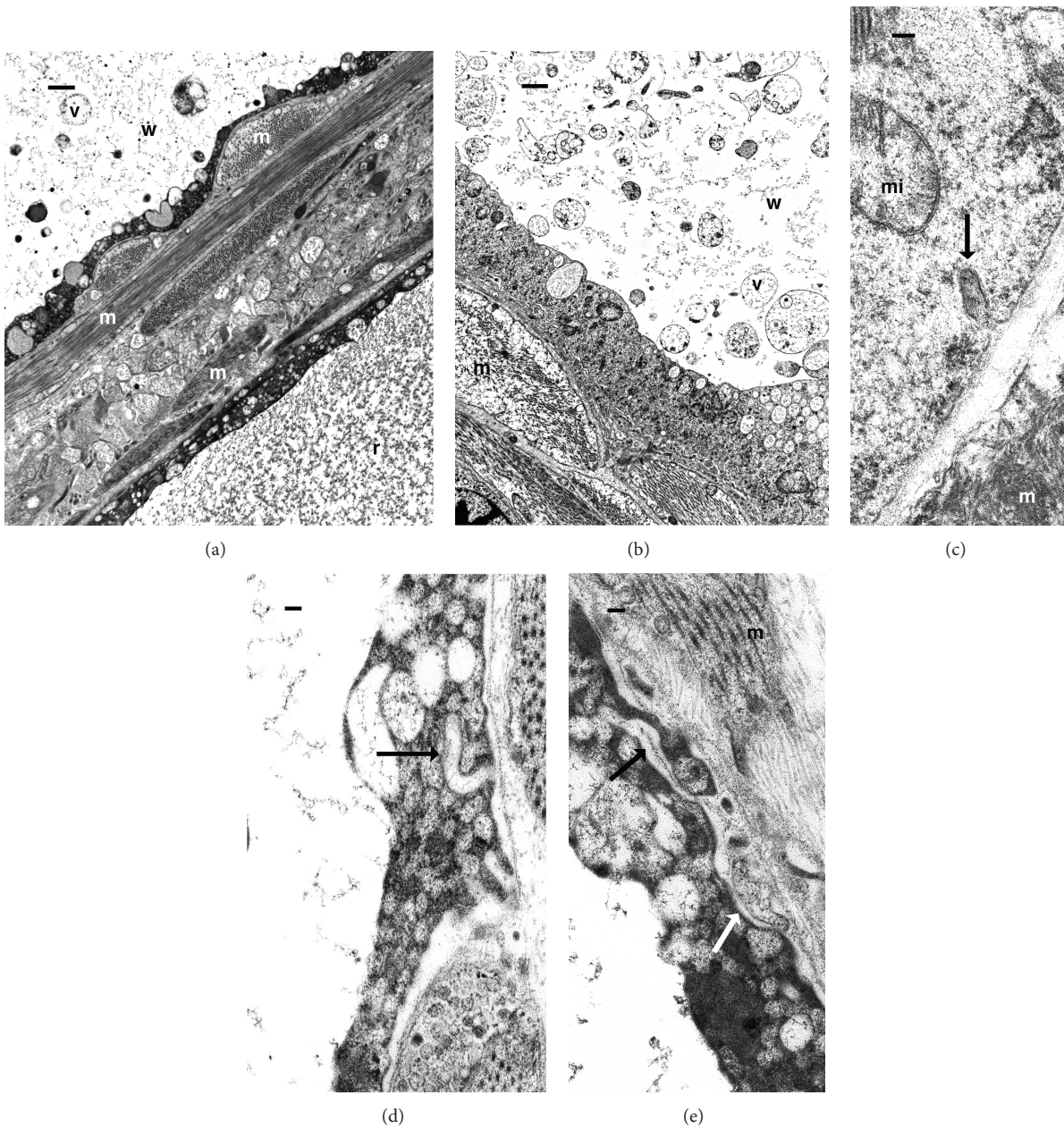
FIGURE 6: White and purple vesicles of *Aplysia parvula*. (a) Adjacent white (lumen filled with vacuoles and filamentous material) and purple (lumen of electron dense, granulate material) vesicles separated by muscle. (b) White vesicle with peripheral cytoplasmic layer surrounding a lumen filled with low electron dense, filamentous material and small vacuoles. ((c)–(e)) The cell membrane of white vesicles is infolded (arrows) into the peripheral cytoplasmic layer and these invaginations contain low electron dense, filamentous material. m, muscle; mi, mitochondrion; r, purple vesicle; v, vacuole; w, white vesicle. Scale bars: (a) and (b) = 1 μm; (c)–(e) = 100 nm.

4.3.2. Synthesis. Where is the ink protein synthesized? Prince et al. [9] and Prince and Johnson [3] suggested two sites for the synthesis of ink protein, the protein vesicles (amber and white) and/or the RER cells. Vesicles have large nuclei (also found in rhodoplast cells and cell bodies [9, 20, 23]) but when they mature, the nucleus lacks a nucleolus [9]. Nucleoli are essential for ribosome formation and, therefore, protein synthesis [24]. In addition, the peripheral matrix of cellular material in both amber and white vesicles contains scant rer.

Furthermore, the vesicle membrane of several of these species appeared actively involved with the uptake of proteinaceous material (process described below). Amber and white vesicles do not appear, therefore, to have the apparatus for synthesis of abundant protein but do have the apparatus for its uptake and storage.

The RER cell type appears to be the site of synthesis of ink protein. This is based upon an evaluation of ink gland structure in a non-ink-producing species, *D. dolabrifera*,
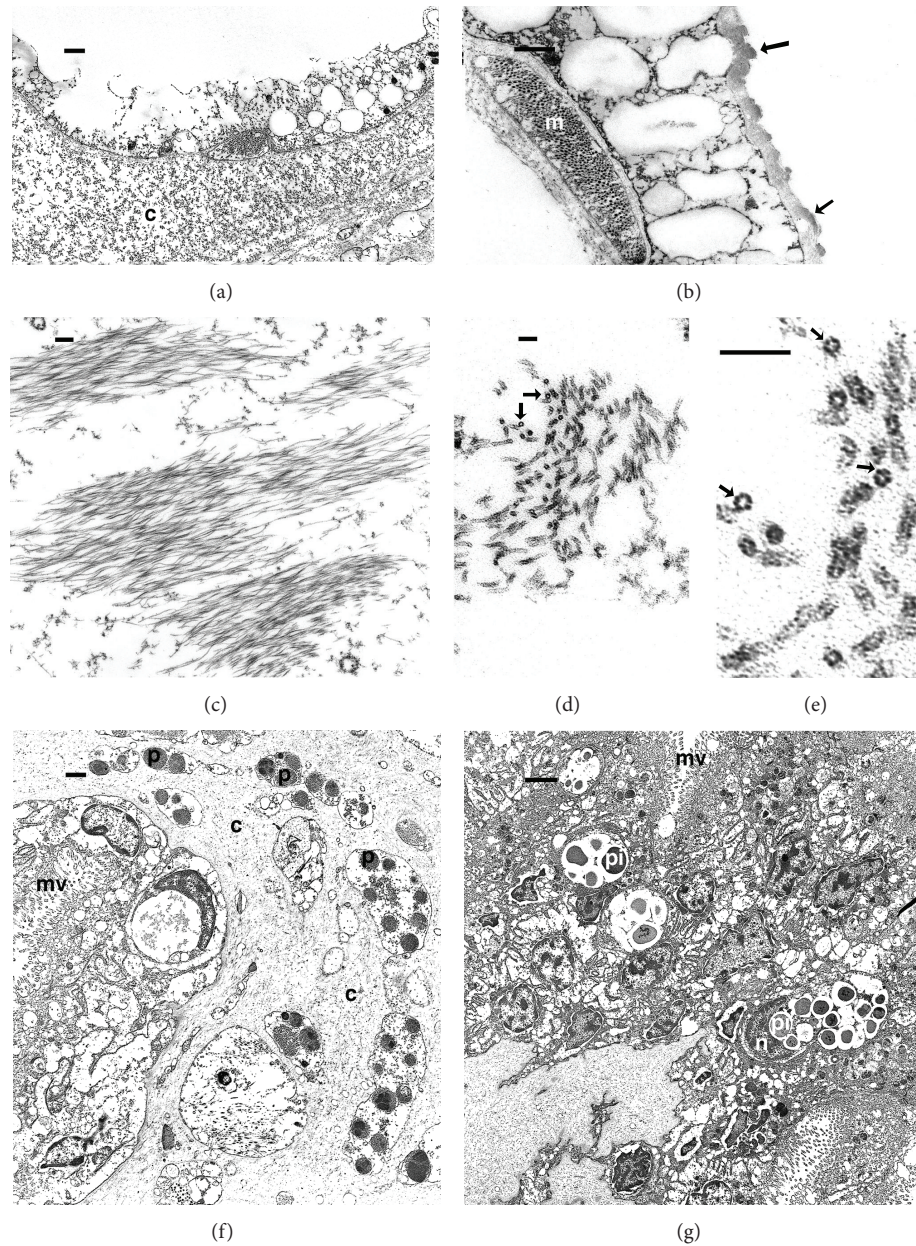
Figure 7: Transparent vesicle of *Dolabrifera dolabrifera* ((a)–(e)) and the epidermis of its ink gland ((f) and (g)). ((a) and (b)) The electron-lucent lumen is surrounded by a thin layer of cytoplasm against which electron dense material (arrows) is deposited. ((c)–(e)) Arrays of filaments with translucent cores (arrows in (d)) and a wall composed of subparticles (arrows in (e)) were occasional found free in the vesicle lumen. ((f) and (g)) Cells below the epidermis containing protein (f) or pigment granules (g). c, connective tissue; m, muscle; mv, microvilli; n, nucleus; p, protein granule; pi, pigment granule. Scale bars: (a) and (b) = 1 μm; (c) = 200 nm; (d) and (e) = 50 nm; (f) = 1 μm; (g) = 2 μm.

compared to that in ink protein releasing sea hares, *A. californica*, *A. juliana*, and *A. parvula*. Even though *D. dolabrifera* has a competent ink gland (having all the necessary cell types for both pigment uptake and storage as well as protein synthesis and storage) it releases no ink, neither the protein nor pigment component. Regarding RER cells in *D. dolabrifera* compared to those in *A. californica*: (1) they occur in a low frequency, (2) they contain minimal rer, (3) the cisternae of rer are seldom expanded, (4) cisternae that are expanded are electron-lucent (i.e., not filled with medium electron dense material), and (5) they lack a nucleolus [3]. We show a similar contrast between the RER cells of *D. dolabrifera* and those of *A. juliana* and *A. parvula*, both of which release both the pigment and protein components of ink.

4.3.3. Why Aplysia californica Releases Ink and Dolabrifera dolabrifera Does Not. This study, coupled with a comparative study of the digestive glands of the same species of sea hare [19], provides the hypothesis for the ability of *Aplysia spp.*

to release ink, both the pigment and protein components, and *D. dolabrifera*'s inability to do so. The digestive gland of both *A. californica* and *D. dolabrifera* has rhodoplast cells and their vacuoles that convert phycoerythrin to PEB. The PEB is then circulated through the sea hares' body by the hemolymph and taken up by pigment cells in the skin. The granulate cells and vesicles in the ink gland of *A. californica* convert PEB to APV, the principal form of pigment in the ink gland [22], allowing uptake. *Dolabrifera dolabrifera*, on the other hand, is apparently unable to convert PEB to APV; thus no pigment is stored nor is colored ink released. The RER cells in the ink gland of *D. dolabrifera* appear to lack the machinery for the synthesis of ink protein. *Aplysia californica* has functional RER cells and the ink protein produced is taken into protein vesicles by pinocytosis (see below). *Dolabrifera dolabrifera*, therefore, can neither form, accumulate nor release ink protein or pigment.

4.3.4. Uptake. How does the protein enter the white or amber vesicles after synthesis by RER cells? The protein synthesized by RER cells appears as low electron dense, filamentous material that fills the distended cisternae of the endoplasmic reticulum (Fig. 6C in [9]). Prince et al. [9] found that vesicle initials had smooth, uninterrupted cell membranes. But the current study found that the cell membrane of vesicle cells (see terminology above) of *A. californica* and *A. parvula* and mature white vesicles of *A. juliana* and *A. parvula* were invaginated to form sacks or extensive blind canal systems. These canals and sacks contained material of medium electron density similar to that in the vesicle itself as well as cisternae of the RER cells. These sacks appeared to become vacuoles filled with filamentous material of medium electron density in the cytoplasmic area of the vesicle cell. The vacuole contents eventually became crystalline, allowing measurement of crystal lattice features, before fusing with the main vacuole of the vesicle. This suggests, therefore, that the protein enters through the vesicle membrane by pinocytosis.

4.3.5. Storage. Johnson et al. [25] found that ink protein is stored in amber vesicles and not in purple vesicles of *A. californica* but mentioned that clear vesicles lacked escapin. No study has addressed what type of vesicle is involved with ink protein storage in other sea hare species; is it stored in several vesicle types or is it restricted to a particular type of vesicle as might be the case for *A. californica*?

We found crystalline (and evidence of noncrystalline, hydrated) protein only in amber vesicles of *A. californica*, the white vesicles of *A. juliana* and *A. parvula,* and subdermal cells in the ink gland of *D. dolabrifera* (Table 1). Protein was not found in clear vesicles of *A. californica* or transparent vesicles of *D. dolabrifera*. Ink protein appeared, therefore, to be stored only in amber or white vesicles and no other type.

Several lines of evidence suggested that white and amber vesicles were essentially the same vesicle type: (1) their protein appeared to be similar (lattice dimensions were not significantly different); (2) they were ultrastructurally similar; and (3) the color difference could be due to an association of different chemical groups with the protein [25]. Since the ink protein is apparently synthesized by the RER cells and not the vesicles themselves [3, 9] this might be where the different chemical groups were attached and not added by the vesicles. We propose the term protein vesicle to include both amber and white vesicles (Table 1).

The structure of the ducts from amber vesicles appeared different from those from purple vesicles. Cells lining the duct from amber vesicles had few microvilli while numerous microvilli were produced by cells lining the lumen of ducts from purple vesicles (Figure 3(b) versus Fig. 6B in [9]).

4.4. Other Vesicles of the Ink Gland. Both clear vesicles of *A. californica* and the transparent vesicles of *D. dolabrifera* share several traits. Both vesicle types have a similar ultrastructure (Table 1; a muscle wall surrounded the vesicle membrane enclosing a thin layer of vacuolated cytoplasm covered by an electron dense layer, the majority of the vesicle interior being electron-lucent). Clear vesicles are transparent in transillumination [9] and, therefore, would be transparent in appearance if not for the numerous highly colored vesicles that are adjacent to them (see Fig 1A in [3]). Neither clear nor transparent vesicles store protein or pigment. Based upon their similar ultrastructure and appearance we suggest that the name for the clear vesicle type in *A. californica* be changed to transparent vesicle (Table 1).

Transparent vesicles of both *A. californica* and *D. dolabrifera* contained unusual inclusions. Those of *A. californica* had parallel stacks of sheets; each sheet had the ultrastructural appearance of a membrane but with approximately half the thickness [24]. These stacks were located in vacuoles in cytoplasmic strands within the vesicle. *Dolabrifera dolabrifera* had arrays of tubes with clear cores that occurred within the interior of the vesicle. Each tube, approximately 11 nm in diameter and composed of 7 subunits, was smaller than microtubules (25 to 30 nm diameter) and nearly equivalent in diameter to intermediate filaments (8–12 nm), but unlike intermediate filaments they had a translucent core [26, 27]. The function of both types of inclusions is unknown; neither inclusion was found in other vesicle types.

Adult sea hares have a conspicuous coloration but also have developed a full array of defense mechanism against predation, including inking [1, 28]. The maturation of these active defenses eliminates the need to be inconspicuous and perhaps this development advertises this defensive posture.

4.5. Opaline and Ink Glands. Both opaline and ink from their respective glands are involved with antipredator defense including such mechanisms as phagomimicry, antifeedants, escape, and enhanced grooming (the latter in spiny lobsters [29]). In addition, white ink has been frequently confused for opaline and our study found, furthermore, that both white ink and opaline consist of protein, not pigment. The protein in ink, however, is made in the RER cells surrounding the protein release vesicles (white or amber) but the release vesicle itself appears to synthesize the protein in the opaline gland (for a review of the opaline gland, see [29]).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

References

[1] P. M. Johnson and A. O. D. Willows, "Defense in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult," *Marine and Freshwater Behaviour and Physiology*, vol. 32, no. 2-3, pp. 147–180, 1999.

[2] P. M. Johnson, *Multi-component chemical defense in sea hares (Gastropoda: Opisthobranchia): antipredator compounds act as both honest and deceptive signals to multiple predator species [Ph.D. thesis]*, Department of Zoology, University of Washington, 2002.

[3] J. S. Prince and P. M. Johnson, "Ultrastructural comparison of *Aplysia* and *Dolabrifera* ink glands suggests cellular sites of antipredator protein production and algal pigment processing," *Journal of Molluscan Studies*, vol. 72, no. 4, pp. 349–357, 2006.

[4] K. M. Jörger, I. Stöger, Y. Kano, H. Fukuda, T. Knebelsberger, and M. Schrödl, "On the origin of Acochlidia and other enigmatic euthyneuran gastropods, with implications for the systematics of Heterobranchia," *BMC Evolutionary Biology*, vol. 10, article 323, 2010.

[5] R. R. Troxler, G. D. Offner, and T. R. Capo, "Structural studies on aplysioviolin," *Biological Bulletin*, vol. 161, p. 339, 1981.

[6] M. Yamazaki, K. Kimura, J. Kisugi, and H. Kamiya, "Purification of a cytolytic factor from purple fluid of a sea hare," *FEBS Letters*, vol. 198, no. 1, pp. 25–28, 1986.

[7] M. Yamazaki, K. Kimura, J. Kisugi, K. Muramoto, and H. Kamiya, "Isolation and characterization of a novel cytolytic factor in purple fluid of the sea hare, *Aplysia kurodai*," *Cancer Research*, vol. 49, no. 14, pp. 3834–3838, 1989.

[8] R. MacColl, J. Galivan, D. S. Berns, Z. Nimec, D. Guard-Friar, and D. Wagoner, "The chromophore and polypeptide composition of *Aplysia* ink," *Biological Bulletin*, vol. 179, no. 3, pp. 326–331, 1990.

[9] J. S. Prince, T. G. Nolen, and L. Coelho, "Defensive ink pigment processing and secretion in *Aplysia californica*: concentration and storage of phycoerythrobilin in the ink gland," *Journal of Experimental Biology*, vol. 201, no. 10, pp. 1595–1613, 1998.

[10] M. Kamio, L. Nguyen, S. Yaldiz, and C. D. Derby, "How to produce a chemical defense: structural elucidation and anatomical distribution of aplysioviolin and phycoerythrobilin in the sea hare *Aplysia californica*," *Chemistry & Biodiversity*, vol. 7, no. 5, pp. 1183–1197, 2010.

[11] J. F. Aggio and C. D. Derby, "Hydrogen peroxide and other components in the ink of sea hares are chemical defenses against predatory spiny lobsters acting through non-antennular chemoreceptors," *Journal of Experimental Marine Biology and Ecology*, vol. 363, no. 1-2, pp. 28–34, 2008.

[12] M. Nusnbaum and C. D. Derby, "Effects of sea hare ink secretion and its escapin-generated components on a variety of predatory fishes," *Biological Bulletin*, vol. 218, no. 3, pp. 282–292, 2010.

[13] H. Yang, P. M. Johnson, K.-C. Ko et al., "Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*," *The Journal of Experimental Biology*, vol. 208, no. 18, pp. 3609–3622, 2005.

[14] H. Kamiya, K. Muramoto, R. Goto, M. Sakai, Y. Endo, and M. Yamazaki, "Purification and characterization of an antibacterial and antineoplastic protein secretion of a sea hare, *Aplysia juliana*," *Toxicon*, vol. 27, no. 12, pp. 1269–1277, 1989.

[15] V. M. M. Melo, A. M. Fonseca, I. M. Vasconcelos, and A. F. F. U. Carvalho, "Toxic, antimicrobial and hemagglutinating activities of the purple fluid of the sea hare *Aplysia dactylomela* Rang, 1828," *Brazilian Journal of Medical and Biological Research*, vol. 31, no. 6, pp. 785–791, 1998.

[16] V. M. M. Melo, A. B. G. Duarte, A. F. F. U. Carvalho, E. A. Siebra, and I. M. Vasconcelos, "Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare, *Aplysia dactylomela* Rang, 1828," *Toxicon*, vol. 38, no. 10, pp. 1415–1427, 2000.

[17] J. Rajaganapathi and K. Kathiresan, "Heparinase in purple fluid of the sea hare, *Bursatella leachii*," *Current Science*, vol. 82, pp. 264–266, 2002.

[18] D. Butzke, N. Machuy, B. Thiede, R. Hurwitz, S. Goedert, and T. Rudel, "Hydrogen peroxide produced by *aplysia* ink toxin kills tumor cells independent of apoptosis via peroxiredoxin I sensitive pathways," *Cell Death and Differentiation*, vol. 11, no. 6, pp. 608–617, 2004.

[19] J. S. Prince and P. M. Johnson, "Role of the digestive gland in ink production in four species of sea hares: an ultrastructural comparison," *Journal of Marine Biology*, vol. 2013, Article ID 209496, 5 pages, 2013.

[20] L. Coelho, J. S. Prince, and T. G. Nolen, "Processing of defensive pigment in *Aplysia californica*: acquisition, modification and mobilization of the red algal pigment, r-phycoerythrin by the digestive gland," *Journal of Experimental Biology*, vol. 201, no. 3, pp. 425–438, 1998.

[21] J. S. Prince and D. Young, "Ultrastructural study of skin coloration in *Aplysia californica*," *Bulletin of Marine Science*, vol. 86, no. 4, pp. 803–812, 2010.

[22] M. Kamio, T. V. Grimes, M. H. Hutchins, R. van Dam, and C. D. Derby, "The purple pigment aplysioviolin in sea hare ink deters predatory blue crabs through their chemical senses," *Animal Behaviour*, vol. 80, no. 1, pp. 89–100, 2010.

[23] R. E. Coggeshall, "A light and electron microscope study of the abdominal ganglion of *Aplysia californica*," *Journal of Neurophysiology*, vol. 30, no. 6, pp. 1263–1287, 1967.

[24] E. Holtzman and A. B. Novikoff, *Cells and Organelles*, Saunders, New York, NY, USA, 1984.

[25] P. M. Johnson, C. E. Kicklighter, M. Schmidt et al., "Packaging of chemicals in the defensive secretory glands of the sea hare *Aplysia californica*," *Journal of Experimental Biology*, vol. 209, no. 1, pp. 78–88, 2006.

[26] J. J. Bozzola and L. D. Russell, *Electron Microscopy*, Jones & Bartlett Learning, Boston, Mass, USA, 1992.

[27] G. N. Gobert and H. Schatten, "Improved ultrastructure of the desmosome-intermediate filament complex in MCF-7 breast cancer cells," *Journal of Electron Microscopy*, vol. 49, no. 4, pp. 539–544, 2000.

[28] T. Carefoot, "Aplysia: its biology and ecology," *Oceanography and Marine Biology Annual Review*, vol. 25, pp. 167–248, 1987.

[29] J. S. Prince, "Opaline gland ultrastructure in *Aplysia californica* (Gastropoda: Anaspidea)," *Journal of Molluscan Studies*, vol. 73, no. 2, pp. 199–204, 2007.