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PROCESSING OF DEFENSIVE PIGMENT IN *APLYSIA CALIFORNICA*: ACQUISITION, MODIFICATION AND MOBILIZATION OF THE RED ALGAL PIGMENT R-PHYCOERYTHRIN BY THE DIGESTIVE GLAND

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Summary

The marine snail *Aplysia californica* obtains its purple defensive ink exclusively from the accessory photosynthetic pigment r-phycoerythrin, which is found in the red seaweeds of its diet. The rhodoplast digestive cell, one of three types of cell lining the tubules of the digestive gland, appears to be the site of catabolism of red algal chloroplasts (rhodoplasts) since thylakoid membranes, including phycobilisome-sized membrane-associated particles, were found within the large digestive vacuoles of this cell. Immunogold localization showed that there was a statistically significant occurrence of the red algal phycobilisome pigment r-phycoerythrin within these rhodoplast digestive vacuoles, but not in other compartments of this cell type (endoplasmic reticulum, mitochondria, nucleus) or in other tissues (abdominal ganglion).

Immunogold analysis also suggested that the rhodoplast vacuole is the site for additional modification of r-phycoerythrin, which makes it non-antigenic: the chromophore is either cleaved from its biliprotein or the biliprotein is otherwise modified. The hemolymph had spectrographic absorption maxima typical of the protein-free chromophore (phycoerythrobilin) and/or r-phycoerythrin, but only when the animal had been feeding on red algae. Rhodoplast digestive cells and their vacuoles were not induced by the type of food in the diet: snails fed green seaweed and animals fed lettuce had characteristic rhodoplast cells but without the large membranous inclusions (rhodoplasts) or phycobilisome-like granules found in animals fed red seaweed.

Two additional cell types lining the tubules of the digestive gland were characterized ultrastructurally: (1) a club-shaped digestive cell filled with electron-dense material, and (2) a triangular 'secretory' cell devoid of storage material and calcium carbonate.

The following model is consistent with our observations: red algal rhodoplasts are freed from algal cells in the foregut and then engulfed by rhodoplast digestive cells in the tubules of the digestive diverticula, where they are digested in membrane-bound vacuoles; r-phycoerythrin is released from phycobilisomes on the rhodoplast thylakoids and chemically modified before leaving the digestive vacuole and accumulating in the hemolymph; the pigment then circulates throughout the body and is concentrated in specialized cells and vesicles of the ink gland, where it is stored until secreted in response to certain predators.

Key words: chemical defense, anti-feedant, phycoerythrobilin pigments, ink, Rhodophyta, red algae, rhodoplasts, opisthobranch mollusc, *Aplysia californica*.

Introduction

Many animals – especially invertebrates – secrete secondary plant products or compounds of their own manufacture as an active defense against predatory attacks (e.g. see Eisner, 1970). Some species accumulate distasteful secondary plant toxins from their diet and distribute them throughout the body as a distasteful, passive chemical defense against predation (Brower and Calvert, 1984). For some, their chemical defense is so effective that they make little effort to hide from predators and they may advertise their defense aposematically (Cott, 1957; Brower, 1984; Eisner, 1970). The marine snail *Aplysia californica* has a chemical arsenal of both active and passive defenses and, like other chemically protected species, it has relatively few predators (reviewed in Nolen *et al.* 1995).

Two of the chemical defense systems of *Aplysia californica* have an origin in its preferred food, red seaweeds (Carefoot, 1987; Winkler, 1969). In particular, halogenated compounds found in red, but not green, algae are accumulated in the snail's skin and internal organs (Winkler, 1961, 1969; Watson, 1973; Stallard and Faulkner 1974*a*,*b*; Kinnel *et al.* 1979) and make the snail distasteful to fish and other predators, thus acting as a passive defense (Ambrose *et al.* 1979; Kinnel *et al.* 1979; Pennings, 1990*a*,*b*). In addition,

30 of the 37 species of *Aplysia* (Nolen *et al.* 1995) secrete a purple ink as an active defense. Like *A. californica*, these species probably concentrate phycoerythrobilin (a modified form of the red algal accessory photosynthetic pigment r-phycoerythrin, found in phycobilisomes located on rhodoplast thylakoid membranes) in vesicles of a specialized gland in the mantle, the ink gland (Chapman and Fox, 1969; MacColl *et al.* 1990). The purple ink, copiously released from this gland during physical encounters with predators, acts as an antifeedant and enhances the ability of the snail to escape from a variety of predators, such as anemones (Nolen *et al.* 1995; Nolen and Johnson, 1998) and probably crabs and lobsters (DiMatteo, 1981, 1982*a*,*b*; Walters *et al.* 1993; Carlson and Nolen, 1997).

While ink consists mainly of r-phycoerythrobilin, the chromophore of r-phycoerythrin (minus its associated protein, see MacColl *et al.* 1990; Troxler *et al.* 1981), it also contains some proteins of a higher molecular mass than the biliproteins associated with phycoerythrin (MacColl *et al.* 1990). Thus, while the pigment component of ink is of algal origin, the protein component – whose anti-predator function is unknown – probably is not. Red algae are the only source of both the ink pigment (Chapman and Fox, 1969) and the distasteful brominated compounds incorporated in the snail's skin (Winkler, 1961, 1969; Watson, 1973; Stallard and Faulkner, 1974*a*,*b*): *Aplysia californica* fed a green seaweed diet lack both types of chemical defenses and are more likely to be eaten in their encounter with a predator (Nolen *et al.* 1995; Pennings, 1990*a*,*b*). Therefore, the acquisition, mobilization, storage and secretion of red algal pigments have significant survival value for *Aplysia*.

Despite the survival value of secondary plant compounds for *A. californica* (Nolen *et al.* 1995), no studies have delineated the mechanisms of acquisition or modification of the plant compound by this herbivore. In particular, we do not know (1) where or how *A. californica* acquires pigment from the red algal cells; (2) where the snail separates the chromophore from the protein component of phycoerythrin; (3) how, and in what form, the red algal pigment gets to the ink gland; (4) what cellular mechanisms are involved in the concentration of the pigment in the vesicles of the ink gland, and finally (5) what function the high molecular mass proteins associated with phycoerythrobilin has in secreted ink. This paper attempts to answer the first three questions, while we address the remaining questions elsewhere (Prince *et al.* 1996; J. S. Prince, T. G. Nolen and L. Coelho, in preparation).

Materials and methods

Growth on different diets

Aplysia californica Cooper, each weighing 0.5 g (wet mass), were placed separately in capped 50 ml clear plastic centrifuge tubes (Corning) with 3 mm holes drilled into the side to provide water exchange. The test tubes were then floated in a 16 l seawater aquarium maintained at 15 °C under continuous illumination (at 35 lx). The snails had been raised to the late juvenile stage [Stage 12; see Kreigstien (1977) for staging criteria] on the red seaweed *Gracilaria tikvahiae* and were then switched to one of three experimental diets: *Ulva lactuca*, a green alga; romaine lettuce; or *Gracilaria tikvahiae*. Initially, six replicates were set up for each diet. Food was provided *ad libitum*, and the test tubes were cleaned daily of feces and food fragments. *Ulva lactuca* was obtained from the Harbor Branch Foundation, Fort Pierce, FL, USA; *Gracilaria tikvahiae* and the snails were acquired from the NCRR *Aplysia* Resource Facility, University of Miami; fresh romaine lettuce was purchased weekly from a local grocery store. For the first 7 days on their particular diet, the snails were de-inked twice per day. At the end of this period, only snails on the red algal diet continued to release ink if disturbed. The wet mass of the snails was measured periodically thereafter until the twentieth day, at which time individuals from each diet group were prepared for electron microscopy.

Electron microscopy

For electron microscopy, snails (3–3.4 g) were anesthetized by injection of isotonic $MgCl_2$ (1 ml g^{-1} wet mass) into the hemocoel. An incision along the muscular foot exposed the digestive system. The digestive gland was removed, fixed in 2.5 % glutaraldehyde in half-strength Millonig's phosphate buffer, pH 7.3, for 2 h at 4 °C, post-fixed in 1 % OsO_4 for 1.5 h, dehydrated in an ethanol series with *en-bloc* staining with uranyl acetate at the 50 % step, and then embedded in Spurr's resin. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips 300 electron microscope at 60 kV. The general ultrastructure of the digestive gland and diverticula was also provided from tissue collected from 250 g snails grown on red algae under conditions similar to those of the growth experiments above. Thick sections (1 µm) provided nuclear size (major length) and cell length and width measurements for various cell types.

The intracellular localization of elemental calcium was determined by thin section analysis using a link energy dispersive spectroscopy (EDS) system ancillary to a Philips 300 transmission electron microscope.

Immunogold electron microscopy

The digestive glands from 250 g snails grown on red algae were prepared for electron microscopy and embedded either in Spurr's resin, as described above, or in LR White resin (without osmium post-fixation or *en-bloc* staining), which was polymerized at 50 °C. Thin sections were caught on nickel grids and immunolabeled with mouse monoclonal anti-phycoerythrin antibody (=anti-PE; Sigma catalog no. P9669) as the primary antiserum (at a dilution of 1:25); this was followed by goat anti-mouse IgG gold (10 nm) conjugate (Sigma) as the secondary antiserum (at a dilution of 1:50). The incubation procedure was slightly modified from that described by Larsson (1979) and Theodosis *et al.* (1986*a*,*b*): sections from Spurr's-embedded tissue were pretreated with

a saturated solution of metaperiodate both to restore tissue antigenicity by removing unreacted osmium and to enhance the hydrophilic nature of the resin (Bendayan and Zollenger, 1983; Causton, 1984; Van den Pol, 1991). Random sections from Spurr's-embedded digestive tissue were selected, and 12 micrographs were taken (at 12 500× magnifications) of each of the various organelles of the rhodoplast cell (rhodoplast vacuole, mitochondria, nucleus and endoplasmic reticulum). For LR-White-embedded rhodoplast digestive cells, six micrographs were taken of the rhodoplast vacuoles and four micrographs were taken of each of the other organelles and analyzed quantitatively by standard stereological methods (Bozzola and Lonnie, 1992). Briefly, a double square lattice grid was superimposed over the negatives of the micrographs and viewed through a dissection microscope at 10× power. Grid points (hits) falling on top of the various organelles were counted, along with the number of gold particles within each organelle. An estimation of the number of gold particles per profile area of organelle in the section was obtained using the following equation (Griffiths and Hoppeler, 1986):

$$N = G/(pd^2)\,, \qquad (1)$$

where N is the number of gold particles per profile area of organelle, G is the number of gold particles counted over a particular organelle, p is the number of grid points (hits) falling on the organelle and d is the distance between grid points (in μm).

Several control experiments were performed to assess the specificity of the immunostaining and the quality of the immunocytochemical method of phycoerythrin (PE) detection. These included: (1) omission of the primary antiserum (no anti-PE; N=12 micrographs); (2) anti-PE immunostaining of abdominal ganglia from the snail (N=4); (3) anti-PE immunostaining of areas of sections devoid of any tissue (Spurr's resin, N=4); (4) the use of a different primary antibody (mouse anti-glutamate, N=12) on the digestive gland; (5) control mouse ascites fluid obtained from BALB/c mice (Sigma) (N=12); (6) liquid-phase adsorption of the anti-PE with r-phycoerythrin (Sigma), with and without 1 mg ml^{-1} and 2 mg ml^{-1} poly-L-lysine (carried out at 4 °C for 24 h under agitation and then centrifuged at 100 ***g***; N=4, for each); (7) solid-phase adsorption of anti-PE with r-phycoerythrin [conducted on cyanogen-bromide-activated Sepharose 4B beads (Pharmacia) at 4 °C for 24 h under agitation, and then centrifuged at low speed; N=4]. For controls, 4–7 above, various dilutions (ranging between 1:5 and 1:75) were tested, but in all cases a dilution of 1:25 was found to be optimal. We calculated the absolute labeling for PE by subtracting the non-specific binding of the secondary antibody (i.e. no anti-PE) and the non-specific binding of the primary antibody (i.e. the average of the PE absorption controls, 6 and 7 above).

The localization resolution of the antigen (PE) is dependent on the spatial resolution of the relatively large secondary antibody (see Results). The spatial resolution of the secondary antibody is the distance between the center of the gold particle and the epitope recognized by the anti-PE. Spatial resolution was calculated as:

length of antibody (16 nm)

+ diameter of gold particle (10 nm) = 26 nm . (2)

For this antibody, see Wolf (1993).

Hemolymph

Approximately 10–15 ml of hemolymph from 100–150 g (wet mass) animals fed red algae or romaine lettuce was removed by hypodermic syringe from the posterior third of the hemocoel following cold anesthesia. Chilling the animal to 4 °C for 20 min anesthetized it sufficiently to prevent ink secretion from the ink gland and contamination of the sample. The optical absorption spectrum between 450 and 600 nm was read against a seawater blank (Bausch and Lomb Spectronic 2000) immediately after centrifugation or after storage at −80 °C.

Statistical comparisons

Before analyzing experimental effects, we ran tests of skewness and/or homogeneity of variances to determine whether parametric statistical tests were valid (Sokal and Rohlf, 1981). Where they were not, we employed appropriate non-parametric statistical tests (e.g. Kruskal–Wallis tests) instead (Krauth, 1988). When multiple comparisons were performed, significance levels were adjusted using a Bonferroni method (Krauth, 1988). Unless otherwise indicated, all significance levels reported are two-tailed, and values reported are means ± S.E.M. Statistical tests were performed using InStat 2.03 for Macintosh (GraphPad Software).

Results

Growth and diet

All three groups of animals grew on their respective diets (two-factor, repeated-measures analysis of variance, ANOVA, on $\log_{10}$-transformed masses: $F_{2,70}$=232.23, P<0.0001), indicating that each food met at least minimal nutritional requirements for juvenile *A. californica*. However, there was a significant effect of diet on growth ($F_{2,14}$=6.13, P<0.0123): a *post-hoc* test showed no difference in the wet masses of animals in the three diet groups up to the end of the de-inking period (day 7), just before the start of the growth experiment (see Fig. 1). There was no statistically significant difference in the wet masses of snails fed the red alga *Gracilaria* or the green alga *Ulva* over most of the duration of the experiment (Fig. 1). In contrast, snails fed red algae were statistically significantly heavier than those fed romaine lettuce, but only over the last week of the experiment (Fig. 1). However, there were no significant differences in growth rate (mass change per day) between any of the three diet groups over the last measurement interval (day 17–20: one-way ANOVA, $F_{2,17}$=3.3865, P>0.06). The lower body mass of the group fed romaine lettuce suggests that food-handling efficiency varied with diet and with the size of the radula (see Discussion).

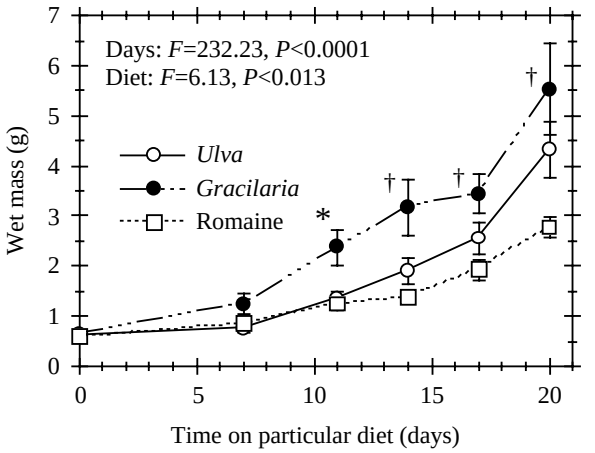

Fig. 1. Growth (wet mass; mean ± S.E.M.; N=6 for all diets) of *Aplysia californica* fed *Gracilaria tikvahiae*, *Ulva lactuca* or romaine lettuce. The animals gained 5–10 times their initial mass over the 3 week trial; diet had a significant effect on growth. The results of a two-factor repeated-measures ANOVA on $\log_{10}$-transformed masses are shown. The outcomes of selected pairwise Bonferroni multiple-comparison tests (a criterion value of t>2.8399 detects a difference at P<0.05) are shown as follows: *statistically significant difference between *G. tikvahiae* and *U. lactuca*; †statistically significant difference between *G. tikvahiae* and romaine lettuce. There were no significant differences (P>0.05) between snails fed *U. lactuca* and romaine lettuce.

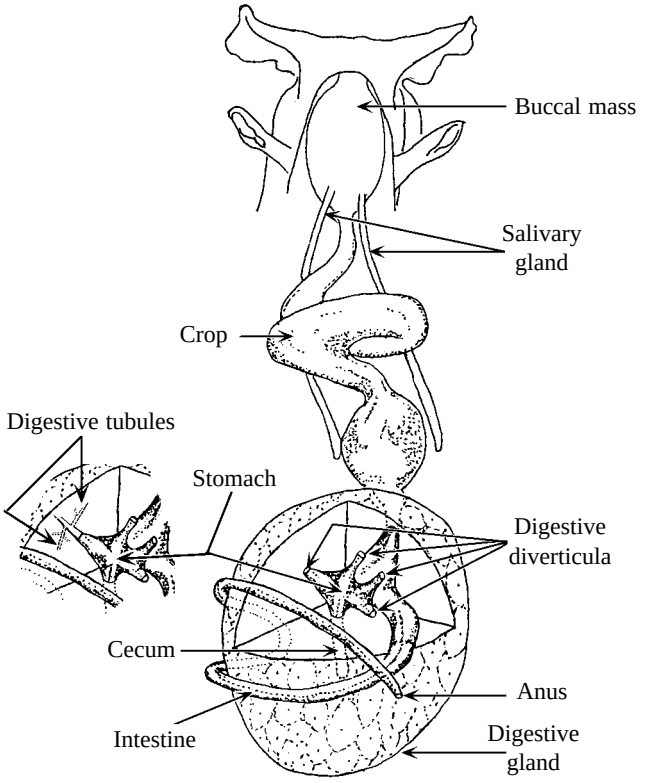

Fig. 2. Diagrammatic dorsal view of the digestive system of *Aplysia californica*. The stomach, digestive diverticula and digestive gland comprise the midgut. The tubules of the four digestive diverticula make up the digestive gland.

Ultrastructure

Red seaweed diet

The stomach of *A. californica* diverges into a number of ducts (Kandel, 1979): (1) the four digestive diverticula or main ducts of the digestive gland that branch off from the stomach; (2) a larger blind duct, the caecum, and (3) the intestine (Fig. 2). The digestive diverticula are lined by two cell types: (1) mucus-secreting cells whose apical surface is involved in mucus droplet formation and/or is extended into numerous, long microvillae (Fig. 3A) and (2) ciliated cells (Fig. 3B). Both cell types are columnar with numerous mitochondria located between the cell apex and the median nucleus.

The digestive diverticula diverge into many small blind-ending tubules (see Fig. 2), which are lined by three types of cells; it is the mass of these tubules that forms what is commonly called the digestive gland (or hepatopancreas) of the midgut. The first cell type that lines the tubules of the digestive diverticula, the club-shaped digestive cell, is elongate with a medially situated nucleus, numerous digestive vacuoles containing electron-dense material and an apical fringe of microvillae that are considerably shorter and less numerous than those covering the cells of the digestive diverticula (Fig. 3C).

The second cell type, Howells' secretory cell (Howells, 1942), is wedge-shaped with little cell surface exposed to the tubule lumen; what little luminal surface the cell has is adorned with only a few short microvillae (Fig. 3C). In contrast to the more numerous digestive cells, secretory cells appear empty and contain only a few, small electron-dense vacuoles and a basally situated nucleus.

The third cell type, the rhodoplast digestive cell, is also wedge-shaped with the narrow luminal surface coated by microvillae (Fig. 4A), but this cell has a wider base and larger nucleus than the club-shaped digestive cell; its nucleus takes up approximately 32 % of the cell volume (Figs 3D, 5). The rhodoplast cell has a dense cytoplasmic matrix consisting of a considerable amount of rough endoplasmic reticulum, mitochondria and several vacuoles (mean diameter of vacuole 8±0.83 μm, N=7). The vacuoles frequently contained a single, whorled structure (Fig. 4B,C). These whorled inclusions appear to be rhodoplasts since they are approximately the same size as the rhodoplasts of *Gracilaria*, ranging in diameter from less than 0.5 to 7.9 μm (N=20). The rhodoplast dimensions for *Gracilaria tikvahiae* are 6.4±0.4 μm by 3.5±0.3 μm, N=16; Prince, 1973). These rhodoplast-like inclusions appear in various stages of digestion (Fig. 4). An energy dispersive spectrum (EDS) analysis did not detect elemental calcium within rhodoplast vacuoles (Fig. 6), so the whorled structures are not the Ca^{2+} accretion bodies seen in other cell types in other species (e.g. Graham, 1938).

As the rhodoplasts are digested, hemidiscoidal granules are released into the vacuole lumen (Fig. 4D). These granules are similar in diameter to the phycobilisomes on the thylakoid

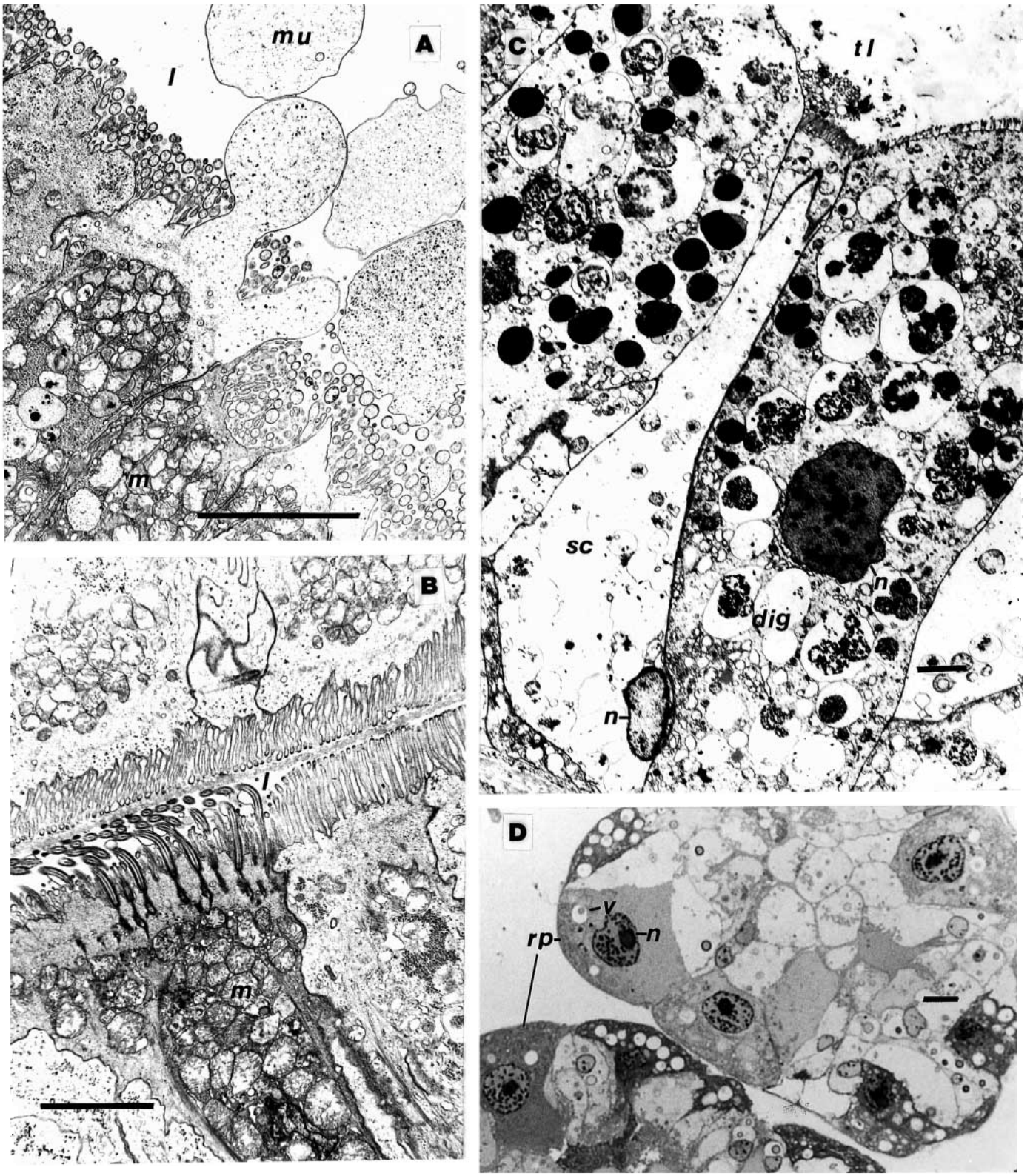

Fig. 3. Digestive diverticula of *Aplysia californica.* Transmission electron micrographs of digestive diverticula (A,B) and digestive tubules (C). Light micrograph of digestive tubule (D). *dig*, digestive cell; *l*, digestive diverticulum lumen; *m*, mitochondrion; *mu*, mucus droplet; *n*, nucleus; *rp*, rhodoplast cell; *sc*, secretory cell; *tl*, tubule lumen; *v*, rhodoplast vacuole. Scale bars, 3 μm.

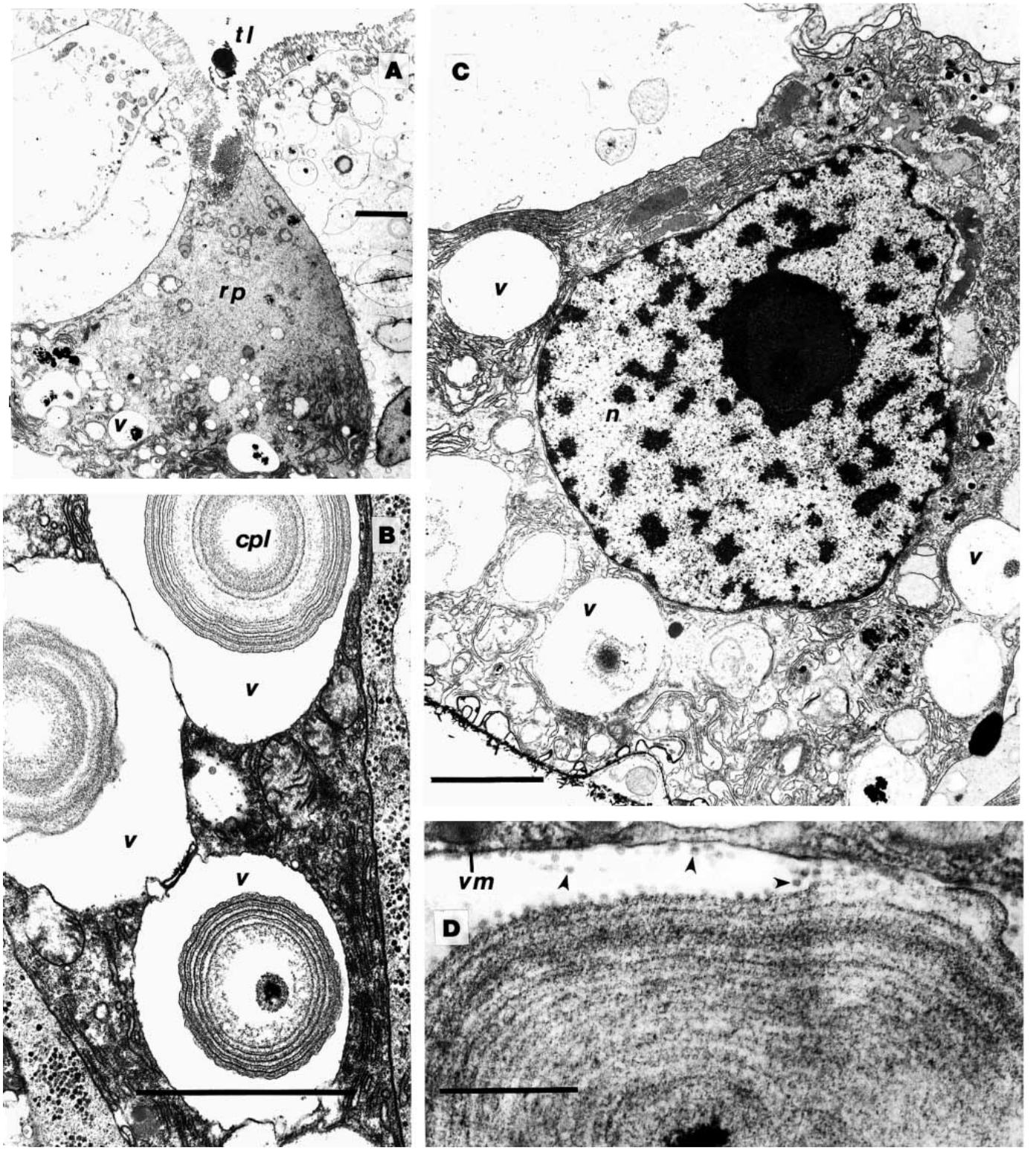

Fig. 4. Transmission electron micrographs of the rhodoplast digestive cell showing cell morphology (A), rhodoplast digestive vacuoles (C) and details of the red algal chloroplasts being digested within these vacuoles (B,D). Arrowheads, phycobilisome; *cpl*, chloroplast; *n*, nucleus; *rp*, rhodoplast cell; *tl*, tubule lumen; *v*, rhodoplast vacuole; *vm*, vacuole membrane. Scale bars, 3 μm, except for D which is 0.5 μm.

membranes of intact red algal rhodoplasts (granules, mean ± 95 % CI, 36.1±0.54 nm, *N*=240 *versus* 30–40 nm reported for phycobilisomes by MacColl and Guard-Friar, 1987). These phycobilisome-like granules are typically found in clusters adjacent to the most recently exposed rhodoplast thylakoid and singly or in small groups against the vacuole membrane

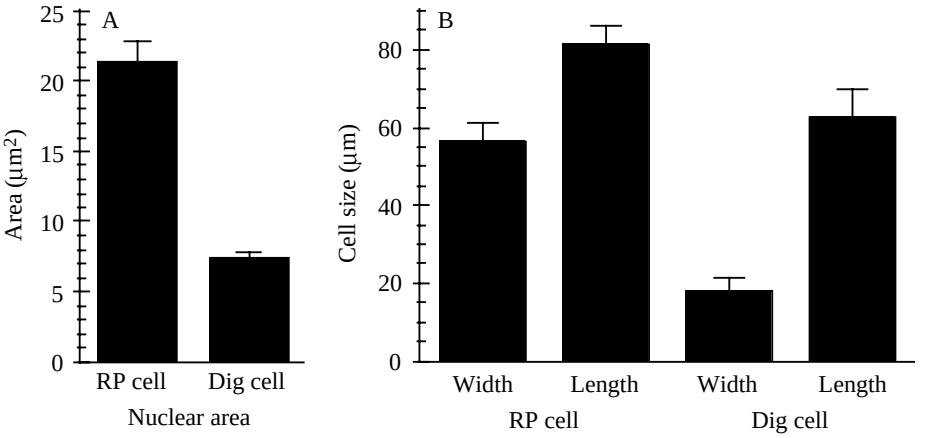

Fig. 5. Rhodoplast digestive cell (RP cell) and digestive cell (Dig cell) traits. (A) Area (mean + S.E.M.) of nucleus (N=15 for each); (B) cell length (N=6 for each) and width (N=6 for each). Significant differences occurred between rhodoplast digestive cell and digestive cell nuclear area (Student's t-test, P<0.001, t=8.9063, d.f.=28) and width (P<0.0001, t=6.6793, d.f.=10), but cell length was not significantly different (P=0.0509, t=2.2179, d.f.=10).

(Fig. 4D). Periodically, the vacuole membrane appears to surround a phycobilisome to form a smaller vacuole, but single phycobilisomes within vacuoles were never seen in the cytoplasm itself.

Green seaweed and lettuce diets

Diet affected the ultrastructure of the cells of the digestive gland. Snails fed the green alga *Ulva* or romaine lettuce had rhodoplast digestive cells with empty vacuoles that lacked chloroplasts, phycobilisome-like granules and other inclusions (Fig. 7A). In addition, both digestive cells and rhodoplast digestive cells had one to several medium osmophilic granules that were not enclosed by a membrane (Fig. 7A). Unlike snails fed marine algae (*Gracilaria* or *Ulva*), the digestive cells of snails fed romaine lettuce had an additional, large, pitted, electron-dense granule that filled most of the cell (Fig. 7C).

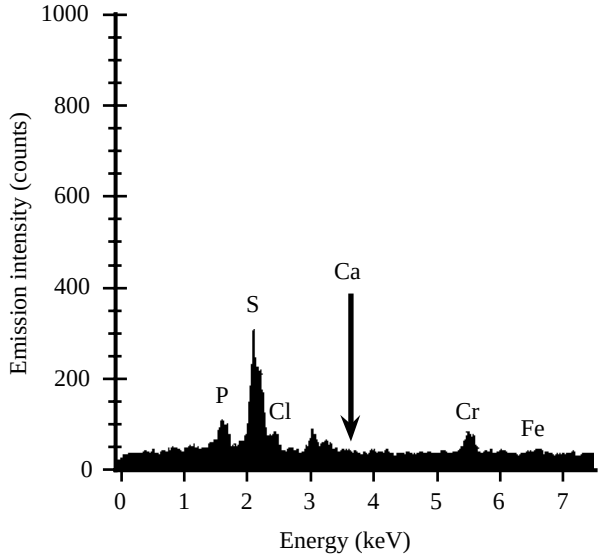

Fig. 6. Energy dispersive X-ray spectrum (EDS) for a spot analysis of an entire rhodoplast digestive vacuole. Emission intensity (counts) is plotted *versus* energy (keV) characteristic for various elements (Ca, calcium, Cl, chloride, Cr, chromium, Fe, iron, P, phosphorus, S, sulfur). Note the absence of any peak for calcium at 3.7 keV.

The granule was contained in a vacuole, which also contained the whorled outlines of material that appeared to coalesce to form the main mass of this granule (Fig. 7D).

Hemolymph

Absorption spectra of hemolymph from snails fed red seaweed had peaks at 493 and 559 nm, similar to peaks reported for phycoerythrin and its free chromophore phycoerythrobilin (approximately 498 and 565–568 nm; MacColl and Guard-Friar, 1987). In contrast, the hemolymph of romaine-fed snails lacked any peaks across the range 450–600 nm (Fig. 8).

Immunocytochemistry

Immuno-gold label for r-phycoerythrin was localized within the rhodoplast digestive vacuoles of the rhodoplast digestive cells (Fig. 7B), with a labeling density of approximately 4 particles μm^{-2} (Fig. 9). Significantly less label was found in the other compartments of the rhodoplast digestive cell, i.e. the nucleus, mitochondria and endoplasmic reticulum, compared with the rhodoplast digestive vacuole. There was no significant difference in the amount of label between these three cell compartments (Fig. 9). Furthermore, relatively little labeling occurred in the abdominal ganglion (see 'Abdom G' of Fig. 10), a tissue not expected to accumulate PE even though the pigment (in some form) circulates in the hemolymph (see above).

Multiple binding of secondary antiserum to the primary antibody (i.e. amplification of the gold label) did not appear to have occurred: gold particles were on average 500±195 nm apart (mean ± 95 % CI; N=10), a distance much greater than the spatial resolution of the secondary antibody (approximately 26 nm, see Materials and methods).

The type of embedding resin (Spurr's *versus* LR White) did not have a significant effect on the amount or distribution of label within the rhodoplast digestive cell (Table 1). Rhodoplast digestive cells in LR White (like those in Spurr's) resin had significantly greater label in the rhodoplast digestive vacuole than in the other cell compartments (see Table 1). Furthermore, there was no significant difference between LR White and Spurr's resins in the amount of label in each compartment. With

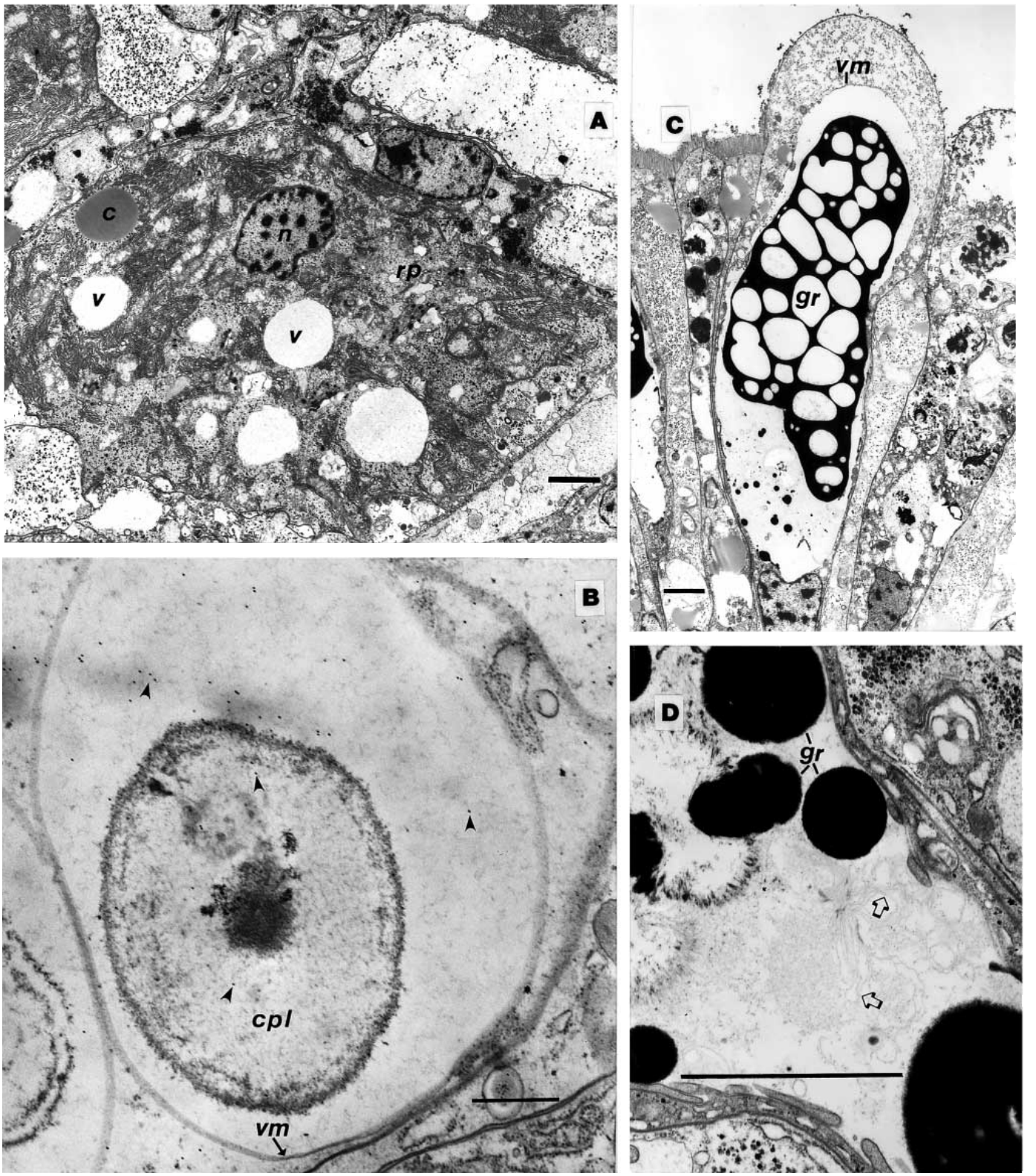

Fig. 7. Transmission electron micrographs of a rhodoplast digestive cell from a romaine-fed snail (A), and (B) immunolabeling for r-phycoerythrin in the rhodoplast digestive vacuole of a snail fed red seaweed. Gold grains (some marked by filled arrowheads) are easily distinguished on negatives viewed and counted with a dissection microscope; see Methods and materials. The label is frequently localized on light osmophilic material in the vacuole. For this entire rhodoplast digestive vacuole, the label is 6 grains μm^{-2}. (C,D) Electron micrographs of electron-dense granules in the digestive cell of romaine-fed snails. Open arrowheads, whorled material; *c*, medium osmophilic granule; *cpl*, chloroplast; *gr*, dense granule; *n*, nucleus; *rp*, rhodoplast cell; *v*, vacuole; *vm*, vacuole membrane. Scale bars, 3 μm, except for B which is 0.5 μm.

Table 1. *Immunogold labeling of rhodoplast digestive cells embedded in either Spurr's or LR White resin*

Organelle	Type of resin	
	Spurr's	LR White
Rhodoplast vacuole	3.97±0.61 (13)	4.98±2.35 (6)
Mitochondria	0.53±0.35 (12)	0.41±0.07 (4)
Nucleus	0.51±0.10 (12)	0.38±0.09 (4)
Endoplasmic reticulum	0.51±0.10 (12)	0.41±0.12 (4)

Cell compartment had a significant effect on the amount of label (ANOVA, $P<0.0001$, $F=6.8887$, d.f.=7.59), with the amount of label being significantly greater in the rhodoplast digestive vacuole in LR White (Student–Newman–Keuls multiple-comparisons test, $P<0.05$, $q \geqslant 4.3353$, d.f.=59), and Spurr's resin ($P<0.001$, $q \geqslant 5.7827$) than that in the other organelles.

No significant difference occurred between the amount of label in organelles in LR White *versus* that in Spurr's resin ($P>0.05$, $q \leqslant 1.4307$).

Values (grains μm^{-2}) are means ± S.E.M. (N).

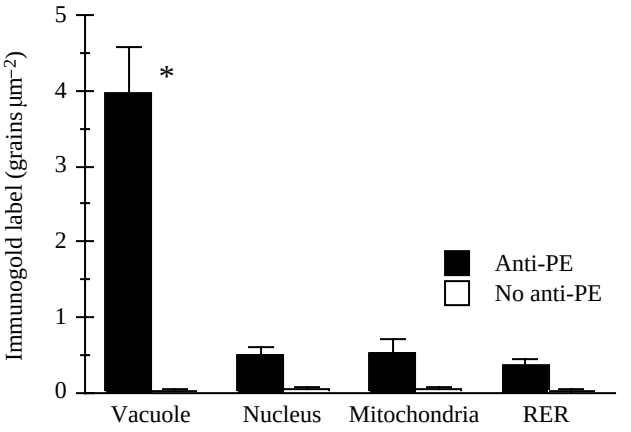

Fig. 9. Immunogold labeling for r-phycoerythrin in the compartments of the rhodoplast digestive cell. Gold-labeling in various cell compartments (organelles) of the rhodoplast digestive cell (mean + S.E.M., N=12 for each organelle) with and without anti-PE (see Materials and methods). RER, rough endoplasmic reticulum. The cell compartment had a significant affect on the amount of label (Kruskal–Wallis nonparametric ANOVA, KW=26.468, $P<0.0001$), with the label for the vacuole being significantly greater (Dunn's multiple-comparisons test, $P<0.001$ for each comparison; asterisk) than that for the other organelles. There was no significant difference in the amount of label between the nucleus, the mitochondria and the RER (Dunn's test, $P>0.05$ for each comparison).

this tissue, Spurr's resin, with osmium and a post-embedding treatment of metaperiodate (see Materials and methods), provided comparable labeling for PE and an improved image compared with that obtained with LR White (with no osmium). No labeling occurred in Spurr's-embedded tissue when the metaperiodate post-embedding treatment was omitted.

Various controls showed that our double-label immunogold localization technique was specific for phycoerythrin (Fig. 10). A small amount of non-specific background labeling was apparent whenever primary antiserum (i.e. anti-PE or anti-Glut) in either its active or inactive (i.e. pre-adsorbed with antigen) form was used. For example, whenever anti-PE was applied, even if pre-adsorbed with antigen (see 'LPh Ads+Lys', 'LPh Ads–Lys' and 'SPh Ads', Fig. 10), small but statistically significant levels of back ground labeling occurred, even over areas of section devoid of cells (see 'Spurr's', Fig. 10). This low level of background labeling probably represents non-specific adherence of (mouse) primary antiserum to the thin sections, since labeling was virtually absent when Anti-PE was omitted (see no 'Anti-PE', Figs 9, 10). Nevertheless, control treatments gave significantly less label than the experimental group (e.g. $P<0.01$, 'Anti-PE', Fig. 10).

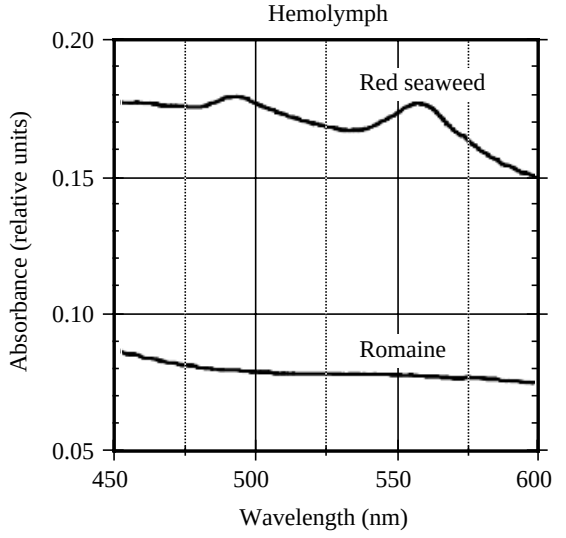

Fig. 8. Absorption spectra (relative absorbance from 450 to 600 nm) of hemolymph from snails fed red seaweed and romaine lettuce.

The average background label attributable to non-specific primary and secondary antibody binding was calculated as follows.

Non-specific primary and secondary antibody binding = **A**

= (the sum of the mean label for four controls)/4

= (1.609 grains μm^{-2})/4 = 0.40 gold grains μm^{-2}.

The non-specific primary and secondary antibody binding is the average of LPh Ads+Lys, LPh ADS–Lys, SPh Ads and Spurr's labeling (see Fig. 10 and Materials and methods).

Non-specific secondary antibody binding = **B**

= (the sum of the mean label for two controls/2)

= (0.35 grains μm^{-2})/2 = 0.17 gold grains μm^{-2}.

The non-specific secondary antibody binding is the average of 'No Anti-PE' and 'Ascites' labeling.

Non-specific primary antibody binding = **C** = **A** – **B**

= 0.23 gold grains μm^{-2}.

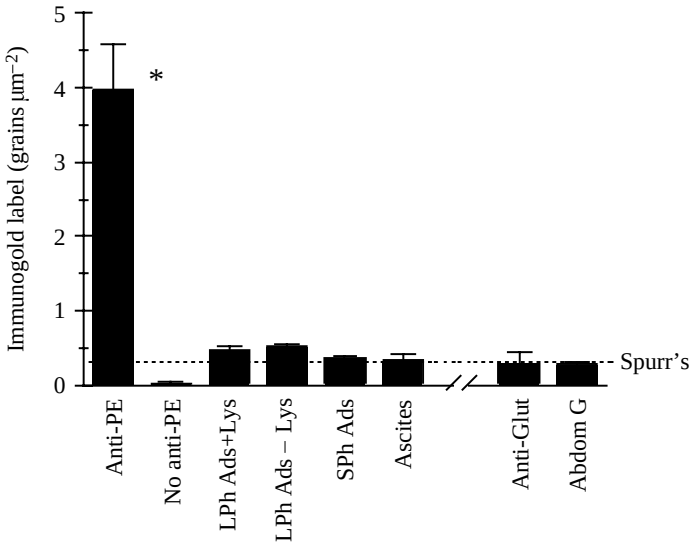

Fig. 10. Immunogold labeling (mean + S.E.M.) for r-phycoerythrin in the rhodoplast digestive vacuole or the abdominal ganglion. Treatments: with anti-PE (N=12), without anti-PE (N=12), liquid-phase adsorption plus lysine (LPh Ads+Lys; N=4), liquid-phase adsorption minus lysine (LPh Ads–Lys; N=4), solid-phase adsorption (SPh Ads; N=4), fluid ascites (Ascites; N=12), glutamate primary (Anti-Glut; N=11), Spurr's resin (Spurr's; N=4), abdominal ganglion (Abdom G; N=4). Treatment had a significant effect on the amount of label (ANOVA, square-root, $\log_{10}$-transformed data, P<0.0001; F=24.723, d.f.=8,59); the amount of label with anti-PE was significantly greater (P<0.01) than for all other treatments (Dunnett multiple-comparisons test; q>2.7320, P<0.05, d.f.=59; asterisk). Except for Anti-Glut, all other treatments had statistically greater labeling than when anti-PE was omitted (Bonferroni multiple-comparison test; P<0.01, t>2.7871, d.f.=59). Labeling between the control treatments (treatments 3–7), the abdominal ganglion and Spurr's were not significantly different (one-way ANOVA, F=1.0747, P=0.3918, d.f.=23).

Absolute PE labeling in rhodoplast vacuole

$$= \text{label with Anti-PE} - \mathbf{A} \text{ or label with Anti-PE} - (\mathbf{B} + \mathbf{C}) = 3.57 \text{ grains } \mu m^{-2}.$$

Absolute PE labeling in rhodoplast cell organelles

$$= \text{(the sum of label in the three organelles with Anti-PE)/3} = 0.52 \text{ grains } \mu m^{-2} - \mathbf{A} = 0.12 \text{ grains } \mu m^{-2}.$$

Subtracting the amount of label attributed to non-specific secondary antibody binding (0.17 grains $\mu m^{-2;}$ see above) as well as the amount of non-specific primary antibody binding (0.23 grains $\mu m^{-2;}$ see above) from that obtained with anti-PE gives an absolute PE-immunogold label of 0.12 grains μm^{-2} for the compartments of the rhodoplast digestive cell *versus* 3.57 grains μm^{-2} for the digestive vacuole, a signal-to-noise ratio of 3.57/0.12 or 29.75:1. Thus, background labeling was relatively insignificant and did not interfere with our localization of PE.

Discussion

Acquisition of the algal pigment phycoerythrin

In *Aplysia californica*, the tubules that diverge from the digestive diverticula make up the digestive gland. These tubules are lined by three morphologically distinct cell types, one of which, the rhodoplast digestive cell, has no comparable description in the literature.

The rhodoplast digestive cell is characterized by large, membrane-bound vacuoles containing red algal chloroplasts – rhodoplasts – in various stages of digestion (Fig. 4A). In addition, rhodoplast digestive cells are wider, have a significantly larger centrally located nucleus and have denser cytoplasm than the two other cell types that line the tubules of the digestive gland. Rhodoplasts were recognized by their size, by the characteristic lamelli of thylakoid membranes and by the phycobilisome-sized particles associated with these membranes (MacColl and Guard Friar, 1987; Sze, 1986). The development of the rhodoplast digestive vacuole *per se* is not a response to diet since it was present in all animals fed any one of the three diets, including romaine lettuce; but the rhodoplast-like inclusions found in the vacuoles were only present in animals actively feeding on red algae.

Griebel's (1993) study of the digestive gland of the opisthobranch *Elysis viridis* showed structures superficially similar to the inclusions we found in rhodoplast digestive vacuoles of *A. californica* (see her Figs 6–9). However, she suggested that they were Ca^{2+} storage structures analogous to those found elsewhere (Graham, 1938). Our EDS analysis of an entire rhodoplast digestive cell vacuole of *A. californica* failed to detect the presence of calcium in the rhodoplast-like inclusions (Fig. 6). In addition, the morphology of these inclusions is otherwise distinct from that of the Ca^{2+} accretion bodies seen in some gastropod secretory cells (where the membranes of Ca^{2+} accretion vacuoles are generally tightly appressed against the calcium granule, the granule itself consists of a few, broadly spaced, thin or thick electron-dense bands in an electron-opaque matrix and the center of mature granule is poorly fixed/embedded and often falls out during preparation; Abolins-Krogis, 1970; Fretter, 1939; Graham, 1938; Walker, 1970).

Our observations suggest that the previously undescribed rhodoplast digestive cell engulfs red algal chloroplasts after they enter the tubules of the digestive gland from the stomach (Fig. 2). The relatively small size of rhodoplasts may be a factor in their ingestion by this particular cell: Fretter (1939) observed that large cell fragments and whole cells (diatoms) are apparently excluded from these tubules. We found that, in snails fed *Ulva* or romaine lettuce, the vacuoles of the rhodoplast cell are present, but empty (Fig. 7A). Rhodoplasts of red algae are typically smaller than those of the belt-shaped chloroplasts of the green alga *Ulva* (Sze, 1986) which, therefore, may not be able to pass into the digestive tubules or cannot be engulfed by the rhodoplast cell itself. Chloroplasts of lettuce appear to be disrupted by the osmotic shock of sea water (J. S. Prince, personal observations) while in the gut and

may, therefore, never reach the digestive gland as whole chloroplasts.

Two additional cell types line the tubules of the digestive gland of *Aplysia californica:* (1) a club-shaped digestive cell that is filled with electron-dense granules and has a few short microvillae projecting into the tubule lumen; and (2) a triangular secretory cell that is devoid of granules (unlike homologous cells in other gastropods; Griebel, 1993).

The digestive cell

These club-shaped cells (Fig. 3C) are described in nearly all previous studies [Fretter, 1939; Gosliner, 1994; Howells, 1942 (equivalent to his absorbing cell); Hyman, 1967], and our observations closely resemble these earlier reports. The suggestion that these cells take up nutrients is supported by our observation that they accumulate electron-dense material when the snail is fed romaine lettuce (Fig. 7C,D). This material was contained within a vacuole which eventually made up the majority of the cell volume. The fate of this material is unknown, but it may represent the accumulation of non-metabolizable lipids from this unnatural (terrestrial plant) food. Prince *et al.* (1993) found that non-metabolizable phospholipids formed similar electron-dense aggregates in vesicles in mouse hepatocytes.

Howells (1942) mentioned that digestive cells both absorb soluble material and excrete vesicles that are pinched off from the cell tip. We never observed vesicle formation by digestive cells, but frequently noted mucus vesicle formation by the columnar cells that line the digestive diverticula.

The secretory cell

This triangular cell wedged between digestive cells (Fig. 3C) is frequently reported to be an excretory cell (Fretter, 1939; Hyman, 1967; Gosliner, 1994), but Howells (1942) termed it a secretory cell. Although the cell may have several functions, we have adopted Howells' (1942) terminology for simplicity.

This cell type, encountered infrequently in *A. californica*, is reported to contain deposits of calcium carbonate in some species of gastropod (Fretter, 1937, 1939, 1941; Gosliner, 1994; Graham, 1938; Hyman, 1967) but not in *A. californica.* The triangular cells in *A. californica* lacked not only calcium carbonate deposits but generally any type of storage (or excretory) vacuoles (see Fig. 3C). Unlike *Aplysia* spp., other gastropod species frequently have a prominent shell and thus this cell may often serve as a site for Ca^{2+} accumulation and storage for shell growth and maintenance. In the case of carnivorous nudibranchs, the calcium is thought to maintain a high pH in the digestive system, which prevents discharge of the nematocysts acquired from their cnidarian prey (Graham, 1938). *A. californica*, however, is a herbivore with a highly reduced shell which contains only a thin remnant of calcium carbonate (Eales, 1921; Hyman 1967). The function (if any) of these triangular cells in *A. californica* remains a mystery, but the absence of calcium carbonate deposits may reflect the reduced calcium regulation of this (nearly) shell-less snail.

Alteration/utilization of phycoerythrin

Immunogold labeling

The defensive ink secreted by *A. californica* is 65 % (dry mass) phycoerythrobilin, some of which still has cystein attached as the only remnant of the biliprotein (Troxler *et al.* 1981). Our ultrastructural study suggests that phycobilisomes are released as the thylakoids of the red algal chloroplast are digested in the rhodoplast digestive vacuole. Phycobilisomes within the rhodoplast digestive vacuole were seldom labeled for anti-PE, perhaps because only free phycoerythrin is recognized by the primary antiserum. Moreover, gold label was dispersed throughout the vacuole lumen but did not occur outside it. Phycobilisomes appeared periodically to become enclosed by an outpocketing of the rhodoplast membrane potentially to form a small vacuole. But small vacuoles with phycobilisomes were never seen (N>100) nor were any small vacuoles within the cytoplasm labeled for r-phycoerythrin. These observations suggest that both the rhodoplast thylakoids and the phycobilisomes are broken down within the rhodoplast vacuole, the phycobilisomes to their respective pigments, phycocyanin, allophycocyanin and phycoerythrin (MacColl and Guard-Friar, 1987).

Phycoerythrin was highly localized to the vacuoles of rhodoplast digestive cells in snails fed red algae. Significantly less phycoerythrin labeling was found in the other cell compartments (nucleus, endoplasmic reticulum, mitochondrion; Fig. 9) of the rhodoplast cell and in another tissue (abdominal ganglion; Fig. 10). Virtually no label was found when the anti-PE was omitted (Fig. 10). While our immunogold labeling technique is specific for phycoerythrin, it produces a low level of background label, even over portions of sections containing just Spurr's resin (see 'Spurr's', Fig. 10). In a related study, we found comparable levels of immunogold labeling for glutamate in the abdominal ganglion; neurons in this part of the nervous system use glutamate as a neurotransmitter. Glutamate is not found at high levels in non-neural tissue (see Fig. 10 'anti-Glut'). Some of the background labeling appears to be due to non-specific adherence of mouse antiserum to thin sections. Adjusting the labeling over the rhodoplast digestive vacuole for background (see Results) gives an average absolute PE label of 0.12 grains μm^{-2} for the compartments of the rhodoplast digestive cell *versus* 3.57 grains μm^{-2} for the digestive vacuole itself, which represents a localization efficiency of approximately 30-fold.

The antigenicity of phycoerythrin is probably altered before it leaves the digestive vacuole because there is little anti-PE staining elsewhere in the cell (Fig. 10) or in other parts of the body ('Abdom G', Fig. 10). The modification in antigenicity is probably due to an alteration to the biliprotein; the epitope for anti-PE appears to be the protein component of phycoerythrin since the fluorescent activity of its bilin chromophore is not altered by antibody binding (A. Cruzan, personal communication, Technical Service, Sigma Chemical Co., for product P9669). Therefore, either the protein is cleaved from the chromophore altogether, leaving only an

attached cystein residue (like the bilin found in the ink gland; see Troxler *et al.* 1981) or the protein is otherwise altered (perhaps by partial digestion by protoeolytic enzymes) so that it is no longer antigenic.

Hemolymph

Winkler (1959) noted that the hemolymph of *A. californica*, normally clear in color, becomes lightly purple after a meal of the red seaweed *Plocamium pacificum*. The absorption spectrum of the hemolymph (maxima at 493 and 559 nm) cannot be used to distinguish between the presence of the free chromophores of phycoerythrin (phycoerythrobilin, phycourobilin) and intact (or slightly altered) phycoerythrin itself since their absorption spectra are nearly identical (absorption maxima of 498 and 565 nm for the bilins and 498 and 568 nm for phycoerythrin, with the height of a shoulder at 545 nm being dependent on the previous light history; MacColl and Guard-Friar, 1987). Nevertheless, our results suggest that modified phycoerythrin and/or the isolated bilin chromophore are present in the hemolymph of animals actively feeding on red algae: absorption spectra of hemolymph from romaine-fed *A. californica* lack evidence of phycoerythrin/bilin. These results suggest that ink pigments travel from the digestive gland to the ink gland in solution *via* the hemolymph, rather than in a bound, chemically inactive (or crystalline) form.

Diet-derived chemical defense

The diet of *A. californica* provides many of its defensive chemicals (Nolen *et al.* 1995). Indeed, pre-metamorphic larval *A. californica* require a red alga to induce settling and metamorphosis (Switzer-Dunlap, 1978; Kriegstein, 1977). Thus, the preference of the snail for red algae serves to ensure its acquisition of unique defensive chemicals unrelated to general nutrition. Carefoot (1967, 1987) found that *A. punctata* grew best on a red algal diet partly because this food provided a greater percentage of amino acids that are better absorbed by snails. But, under *ad libitum* conditions, he found that weight gain could be similar on both green and red algal diets because more green than red algae were eaten. Pennings (1990*a*,*b*) found that, by the time his juvenile snails reached 1–2 g in size (his 'juveniles' were comparable in size to our small adults, 1–2 g), they could grow on the green alga *Ulva* as well as on the red alga *Plocamium*. (The 'recruits' of Pennings were apparently Stage 11 and 12 juveniles according to Kriegstien's (1977) nomenclature and therefore somewhat smaller than those at the start of our feeding experiment). We noted some differences in the initial growth of juveniles (0.5 g) fed on red seaweed *versus Ulva* and romaine lettuce, even under conditions of *ad libitum* feeding. However, after reaching a size of approximately 1.5 g, their growth rates were similar across diets (days 17–20, Fig. 1). Thus, in terms of essential nutrients, both our red and green algae, and even the terrestrial plant romaine lettuce, were adequate to support growth. Anecdotal reports (T. Capo, personal communication) suggest that *A. californica* researchers have had success maintaining fully adult snails on a diet of lettuce for several weeks. We have shown that juveniles and small adults can grow (albeit slowly at first) and thrive on romaine lettuce: we have been able to grow animals to 100 or 200 g on romaine lettuce, and one individual reached approximately 500 g. One practical consequence of these observations is that 'inkless' *A. californica* may be raised on romaine lettuce in the laboratory. However, there may be long-term adverse effects caused by the build-up of non-metabolizable lipids from lettuce in the digestive gland of the snail.

The initial differences in growth that we recorded in our smallest snails (days 7–14, Fig. 1) may have been due to their different food-handling efficiencies for the three diets rather than to food quality, since the growth rates of larger snails on the three foods were similar (Fig. 1). Our observations are consistent with those of Pennings (1990*a*,*b*). In a review of the effect of plant morphology on the feeding of both terrestrial and aquatic herbivores, he suggested that young *A. californica* do not eat certain naturally occurring algae (e.g. *Ulva*, *Codium*, green algae) because of their relatively weak mouth parts. Klinger (1982) found that plant morphology affected the feeding rate of other herbivores (sea urchins): round forms of seaweeds were more rapidly eaten than flat forms. Our animals clearly had some difficulty with broad-leaf foods, i.e. *Ulva* (sometimes called sea lettuce) and romaine lettuce, which affected early growth (Fig. 1). Perhaps the shape, texture or composition of broad-leaf plants generally prevents efficient rasping by the radula in small, but not in more mature, *A. californica*.

Structure and function of the digestive gland of opisthobranchs

There is disagreement in the literature about the function, and even the name, of the digestive gland of opisthobranchs. For example, the midgut of opisthobranchs, and in particular that of *Aplysia*, has been termed the 'liver', the 'hepatopancreas' or simply the 'digestive gland' (Eales, 1921; Stallard and Faulker, 1974*a*,*b*; Watson, 1973). Here, we refer to the bulk of the midgut (the tubules of the digestive diverticula) as the digestive gland. Regarding its function, Eales (1921), Fretter (1937, 1939) and Kandel (1979) suggest that the digestive gland mainly produces enzymes that break down various carbohydrates which then can be absorbed. In comparison, Graham (1938) suggests that the cells of the digestive gland of aeolid gastropods are able to phagocytize cell fragments or particles as well as to secrete various digestive enzymes. Additional reports (Howells, 1942; Stallard and Faulker, 1974*a*,*b*; Winkler, 1969) suggest that the digestive gland of *A. californica* mainly produces digestive enzymes and is involved with the extraction, processing and storage of various toxic compounds (e.g. terpenes, Stallard and Faulkner, 1974*a*). Except for a few reports of phagocytosis by cells in the midgut [e.g. nematocysts in nudibranchs (Graham, 1938), chloroplasts in sacoglossans (Griebel, 1993) and non-food particulates in *A. californica* (Fretter 1939)], most earlier studies are equivocal on whether the digestive gland of *A. californica* normally takes in both soluble and particulate

materials. We found that rhodoplast digestive cells in the digestive gland phagocytize red algal chloroplasts, then digest them in digestive vacuoles and, consequently, modify their associated pigment r-phycoerythrin in the process.

In addition to the confusion about the name and function of this organ, the number of cell types making up the digestive gland is variously reported to be one (Eales, 1921), two (Fretter, 1941; Graham, 1938; Hyman, 1967; Taylor, 1968), three (Fretter, 1939; Gosliner, 1994; Griebel, 1993), four (Fretter, 1939; Howells, 1942; Walker, 1970) or five (Gosliner, 1994). Furthermore, Howells' (1942) detailed description of the cells composing the epithelium of the digestive diverticula of *Aplysia punctata* is probably a description of cells lining the tubules that branch off the four digestive diverticula. Unlike Howells' report, we found that the digestive diverticula (see Fig. 2) are lined by a columnar, ciliated epithelium as well as by secretory cells with numerous long microvillae. Eales (1921) and Fretter (1941) also report a ciliated epithelium lining the digestive diverticula. Furthermore, we found three distinct cell types lining the tubules that diverge from the digestive diverticula. Fig. 2 clarifies our knowledge and the terminology we have applied to the digestive system of *A. californica*.

Acquisition and utilization of the algal pigment phycoerythrin

In conclusion, we have found that specialized cells in the digestive gland of *A. californica*, rhodoplast digestive cells, phagocytize the chloroplasts of red algae, digest them and release phycobilisomes, which are further digested to their pigment complement, mainly phycoerythrin. The chromophore of phycoerythrin is cleaved from some or all of its protein component within the vacuole of the rhodoplast digestive cell, and the modified pigment moves to the hemolymph and is circulated throughout the body. We are currently examining the accumulation of phycoerythrin/phycoerythrobilin in the ink gland (Prince *et al.* 1996), where pigment is accumulated by pinocytosis from the hemolymph and stored in a crystallized form by specialized cells (the granulate cells). Pigment is then mobilized and repackaged in smooth-muscle-enclosed vesicles distributed throughout the ink gland. The muscles of the ink vesicles are activated under central nervous control (Carew and Kandel, 1977), causing ink secretion when the animal is attacked by a predator.

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