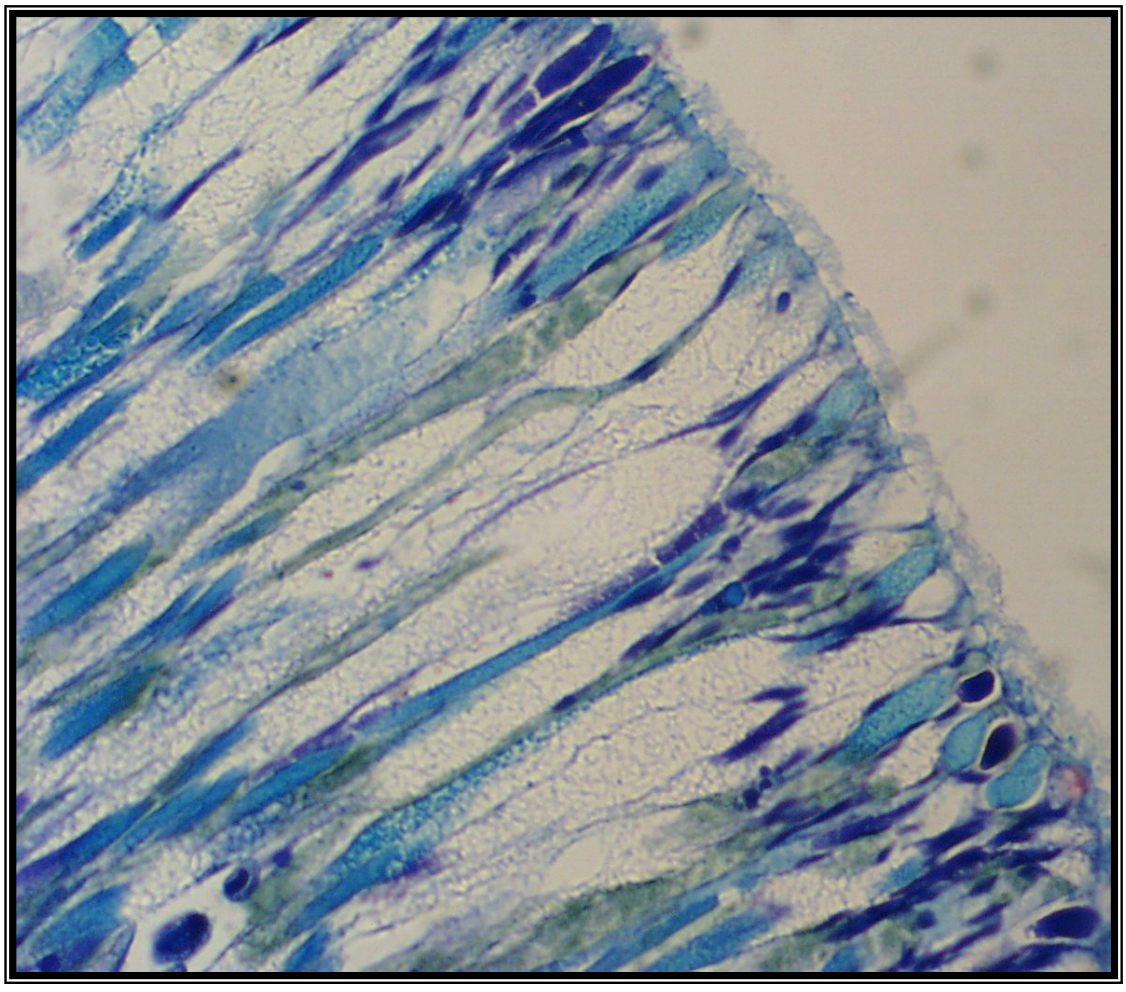
Chapter 5

Evidence for the regulated *de novo* biosynthesis of Tyrian purple in the hypobranchial gland of *Dicathais orbita* (Neogastropoda: Muricidae)



5. 0 Abstract

Investigations into Tyrian purple genesis within hypobranchial glands of muricid molluscs have provided a wealth of information on dye composition and biosynthesis from colourless prochromogens. However, little is known on the origin of the precursor, tryptophan, and regulatory processes governing prochromogen and bioactive intermediate synthesis. As an alternative to radioisotopes, novel histochemical techniques for the demonstration of bromoperoxidase and the brominated prochromogen, tyrindoxyl sulphate, were applied in conjunction with standard stains for tryptophan and arylsulphatase. Two sites of prochromogen synthesis were identified within the hypobranchial gland of the muricid, *Dicathais orbita*. Endocytosed tryptophan sourced from rectum waste appears to be stored within secretory cells of the branchial and rectal hypobranchial epithelium, which upon liberation onto the epithelial surface, is united with bromoperoxidase to form crystals of tyrindoxyl sulphate. Prochromogen synthesis also occurs in the vascular sinus immediately dorsal of the medial hypobranchial gland for storage and secretion by medial epithelial cells. Hydrolysis of tyrindoxyl sulphate and subsequent production of bioactive intermediates appears to be regulated, and only occurs once arylsulphatase is exocytosed onto the surface by adjacent supportive cells. This histochemical approach identified the origin of tryptophan, provided evidence for post-translational precursor bromination, the constitutive and controlled genesis of prochromogens and the regulated synthesis of bioactive intermediates in *D. orbita*. Together these findings strongly suggest the *de novo* synthesis of Tyrian purple

precursors in the Muricidae, which further implies a naturally selected function for the synthesis of these bioactive metabolites.

5. 1 Introduction

Examination of stable isotope incorporation by mass spectrometry and high field NMR has been traditionally employed in determining the biosynthetic origin of secondary metabolites in marine molluscs (Cimino et al., 2004). However, low incorporation rates often render questionable results (Cimino et al., 2004) and labeled precursor diversion into extraneous metabolic pathways (Garson, 1993) can be misleading. A reliance on radioisotope incorporation is also insensitive to the progressively recognized role of symbiotic microorganisms in natural product biosynthesis (Cimino et al., 2004). Furthermore, this technique fails to provide anatomical information, which is particularly useful to subsequent genetic investigations, as natural products are often stored in a different cellular environment to where they are synthesized (Garson, 1993). Although rarely employed, a histochemical approach has the potential to address some of these limitations and ultimately enhance our understanding of secondary metabolite biosynthesis. Histochemical techniques are available for various amino acids, proteins, glycoproteins, carbohydrates, lipids and enzymes (see Thompson 1966). The application of staining reactions for proposed precursors and enzymes can not only establish the primary metabolic origin of natural products, but identify sites of active biosynthesis and qualitatively define the comparative concentration and activity of precursors and enzymes, respectively. The biochemical and morphological properties of associated biosynthetic tissues can also provide information on the transport,

storage and secretion biosynthetic constituents and highlight potential regulatory mechanisms. One highly renowned marine natural product, and an ideal model for histochemical biosynthetic investigations, is Tyrian purple.

Tyrian purple, also known as Royal purple, Shellfish purple or Purple of the Ancients, is an historically important dye obtained exclusively from marine molluscs of the family Muricidae. Evidence for the commercial production of Tyrian purple since the 13th century B.C. (Naegel and Cooksey, 2002) has prompted investigation into the genesis of this dye within muricid hypobranchial glands. In 1909, Friedländer investigated the chemical composition of Tyrian purple from *Murex brandaris*, and elucidated the purple pigment as the brominated indole, 6,6'-dibromoindigo (**5**, Fig. 1). Identification of four prochromogens (Fig. 1) within the hypobranchial gland of *M. trunculus* (Fouquet and Bielig, 1971; Baker, 1974), and a fifth (**1**, Fig. 1) from *Dicathais orbita* (Baker and Sutherland, 1968) followed this discovery. These colourless compounds were found to be 6-bromoindoxyl and indoxyl sulphate salts of choline esters, substituted with methylthio or methylsulphonyl (McGovern and Michel, 1990). Enzymatic hydrolysis of prochromogens by purpurase (arylsulphatase) (Erspamer, 1946) liberates associated choline esters ($\mathbf{X}^+$, Fig. 1) and leads to the formation of yellow indoxyl intermediate precursors. Substituted bromoindoxyl intermediates (e.g. **2**, Fig. 1) are then oxidized to red indoleninones (e.g. **3**, Fig. 1) and dimerize to give green tyriverdin (**4**, Fig. 1) (Christophersen et al., 1978; McGovern and Michel, 1990). External to the mollusc, tyriverdin undergoes photolytic cleavage to yield dimethyl sulphide and 6,6'-dibromoindigo (Baker and Sutherland, 1968).

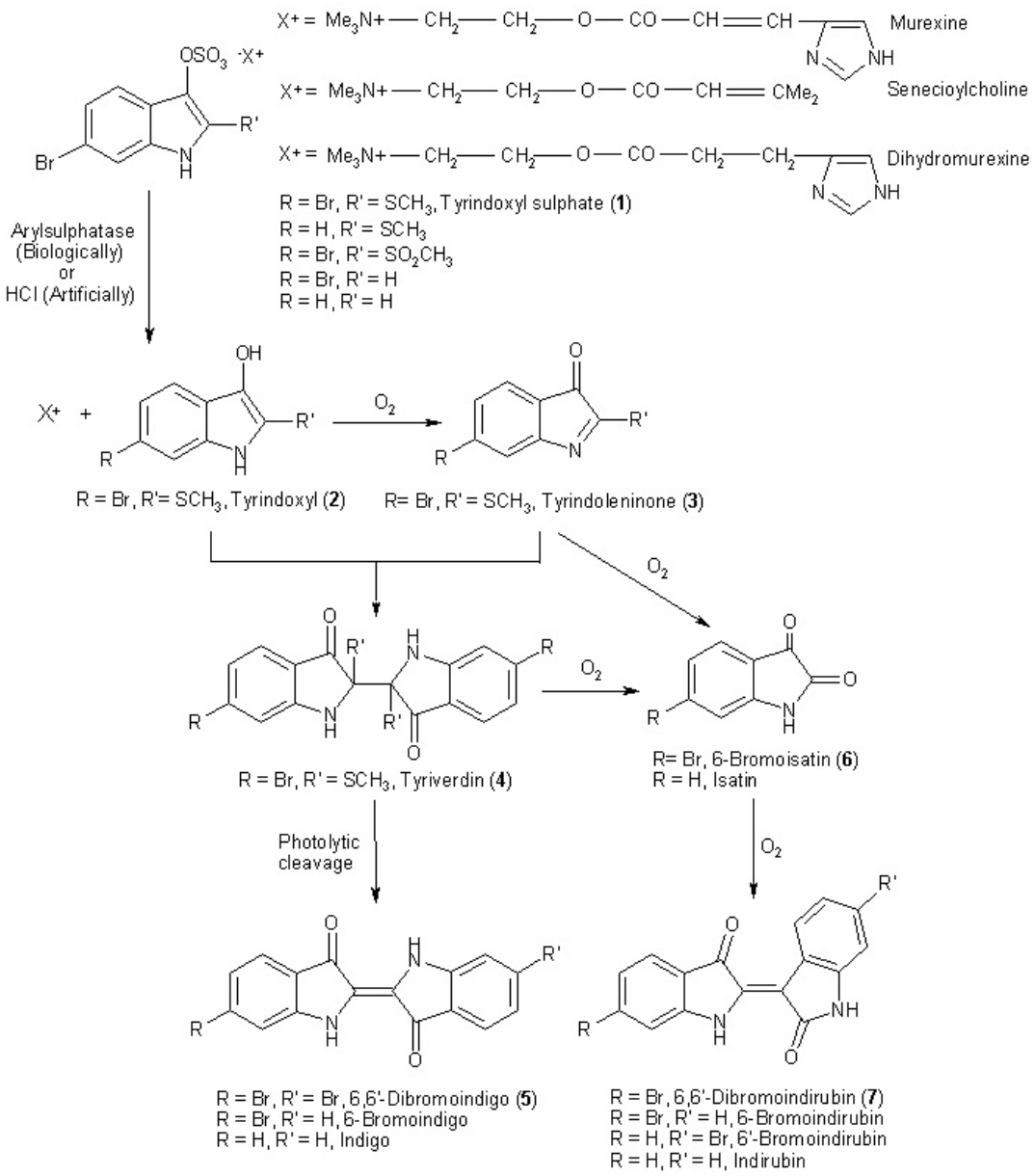


Figure 1. The biosynthesis of Tyrian purple pigments (e.g. **5** and **7**) from brominated (e.g. **1**) and non-brominated indoxyl sulphate prochromogens in the hypobranchial glands of muricid molluscs. Within the hypobranchial gland, hydrolysis by arylsulphatase liberates choline esters ($\mathbf{X}^+$) and catalyzes indoxyl precursor genesis (e.g. **2**), a process which can be replicated artificially by exposure to hydrochloric acid (HCl). This biosynthetic pathway was constructed in accordance with material reviewed in Cooksey (2001a) and Roseghini et al. (1996).

Depending on species prochromogen diversity, dibromoindirubin (**7**), mono- and non-brominated indoles and indirubins may also evolve as dye components (Wouters and Verhecken, 1991; Wouters, 1992; Koren, 1995; Cooksey, 2001a; Cooksey and Withnall, 2001; Karapanagiotis and de Villemereuil, 2006). Despite the wealth of information that exists on the chemical composition of dye pigments and their synthesis from colourless prochromogens, little is known on the biosynthetic origin of prochromogens and the regulatory processes governing genesis.

Indole biosynthesis commences with the essential amino acid tryptophan. Tryptophan synthesis from the phosphorylated sugar, erythrose-4-phosphate, is almost completely restricted to plants (Crawford, 1989; Bentley, 1990; Herrmann et al., 1992). Consequently, *de novo* conversion of dietary derived tryptophan to indole or directly to indoxyl sulphate remains the most plausible explanation for the presence of prochromogens in the Muricidae (Westley et al., 2006). Tryptophan has been detected in hypobranchial secretory cells of the muricids *M. brandaris* (Bolognani-Fantin and Ottaviani, 1981), *M. granulata* (Srilakshmi, 1991) and *P. pansa* (Naegel and Aguilar-Cruz, 2006). However, the origin of this amino acid and the mode of transportation and concentration within these cells have not been addressed. A recent investigation into the histomorphology of *D. orbita* proposed the rectum and lateral hypobranchial secretory cells as the source and site of tryptophan storage, respectively (Chapter 3). It has also been assumed that histochemical demonstration of tryptophan is synonymous with Tyrian purple precursor localization (Naegel and Aguilar-Cruz, 2006). However, it is unlikely that the methods employed (Adams, 1957; Davenport, 1960) would react with indole precursors due to the lack of an available amine functional group (Fig. 2).

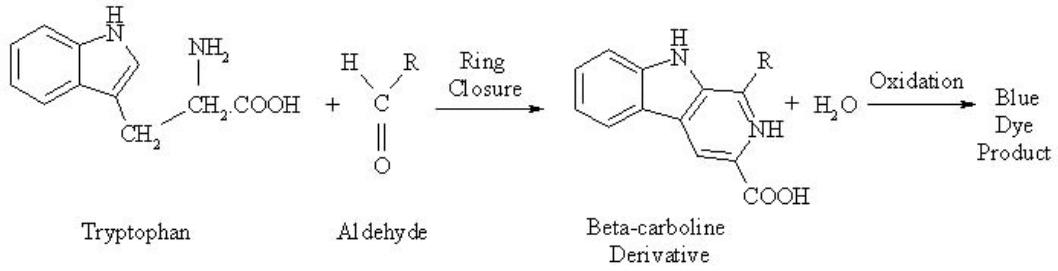


Figure 2. The proposed reaction between the amine functional group (NH_2) of tryptophan and an aldehyde to produce a blue dye in the presence of an oxidizing agent in the p-DMAB-nitrite method for tryptophan (Adams, 1957).

Incorporation of bromine into the 6-position of the indole ring of bromoindoxyl sulphate prochromogens (e.g. **1**), implies enzymatic bromination during Tyrian purple precursor synthesis. Detection of bromoperoxidase activity within hypobranchial gland homogenates of *M. trunculus* (Jannun and Coe, 1987) strongly suggests a capacity for peroxide-induced bromination. To date, bromoperoxidase has only been investigated in one muricid species and the site of precursor bromination within the hypobranchial gland remains unknown.

Hydrolysis of prochromogens into indoxyls and the subsequent formation of intermediates are governed by arylsulphatase and oxygen availability, respectively (Fig. 1). Hypobranchial glands obtained directly from live specimens contain colourless secretions, while those of frozen glands are yellow/green (Baker and Duke, 1974). These observations suggest that prochromogen hydrolysis is regulated by a mechanism which is abolished in autolyzed glands. Erspamer (1946) was able to demonstrate discrete storage of arylsulphatase in the anterior medial hypobranchial gland of *M. trunculus* and *M. brandaris,* and predominance of prochromogens in the posterior. Although this loose compartmentalization may prevent spontaneous

prochromogen cleavage, the coincidence of prochromogens and arylsulphatase in the anterior medial region in *M. brandaris* (Erspamper, 1946), suggests the presence of a more complex mechanism. Furthermore, prochromogen hydrolysis results in the liberation and subsequent synthesis of a suite of compounds (Fig. 1), which may be of selective benefit to the Muricidae (see Westley et al., 2006). The choline esters ($\mathbf{X}^+$), murexine (Erspamer and Dordoni, 1947), senecioylcholine (Whittaker, 1957) and dihydromurexine (Roseghini, 1971), display neuromuscular blocking and nicotinic actions (reviewed in Roseghini et al., 1996) and intermediates, tyrindoleninone (**3**), 6-bromoisatin (**6**) and tyriverdin (**4**) possess cytotoxic properties (Benkendorff et al., 2000; Westley et al., 2006; Vine et al., 2007). The demonstrated activity of these compounds together with the apparent regulation of their synthesis strongly suggests an intricate mechanism for instigating prochromogen cleavage within muricid hypobranchial glands.

D. orbita contains a single prochromogen, tyrindoxyl sulphate (**1**) (Baker and Sutherland, 1968) and consequently, possesses the most simplistic biosynthetic pathway to Tyrian purple. Thus, *D. orbita* is an ideal species for investigating prochromogen biosynthesis and hydrolysis within the Muricidae. Through the application of novel histochemical techniques for the localization of precursors and enzymes required for Tyrian purple synthesis, this investigation aims provide insight into the biosynthetic origin and regulatory mechanisms governing dye synthesis.

5. 2 Methods and materials

A total of 24 female *D. orbita* specimens were collected from the rocky intertidal and subtidal regions of the Adelaide metropolitan coast, Fleurieu and Eyre

peninsulas of South Australia. As hypobranchial gland secretory activity is thought to elevate during the breeding season (Fretter and Graham 1994), prochromogen synthesis and enzyme activity may become more evident during the copulating or egg-laying seasons. Consequently, six females representing each of four reproductive phases were collected over the annual cycles of 2005 and 2006. These phases included; 1) post-reproductive, March; 2) pre-reproductive, early July; 3) copulating, August-September; and 4) egg-laying, late November-December.

The shell of each live specimen was removed by cracking with a vice at the junction of the primary body whorl and spire, and the soft body removed by severing the columnar muscle. The soft body was then transferred to a dissecting tray and submersed in filtered (0.22µm) seawater to reduce osmotic stress. The dorsal mantle and pallial gonoduct were separated from the rest of the visceral mass by an incision along the lateral margins of the columnar muscle. The mantle was then folded back and pinned with the ventral surface facing up. Longitudinal and transverse incisions were made along the junction between the ctenidium and branchial hypobranchial epithelium, and the posterior gonoduct and digestive gland, respectively. Integrity between the hypobranchial gland and gonoduct was maintained to prevent damage to the rectal hypobranchial region and to allow histochemical examination the rectum and rectal gland as possible sites of dietary tryptophan.

Of the 24 specimens, 12 were fixed in 10% neutral buffered formalin (6h), dehydrated through an ethanol series, cleared in chloroform, embedded in paraffin and sectioned at 5µm. Tissue from the remaining 12 specimens was immediately fresh-frozen in O.C.T. compound (Tissue-Tek®) at -20°C, sectioned at 15µm and

affixed onto charged slides (ProSciTech, G311SF-W). Serial transverse sections were obtained from five separate regions (A-E) along the length of the hypobranchial gland (Fig. 3) of all 24 specimens. These regions included, A) the epithelium adjoining the anterior; B) medial and C) posterior capsule gland; and the D) anterior and E) posterior ingesting gland.

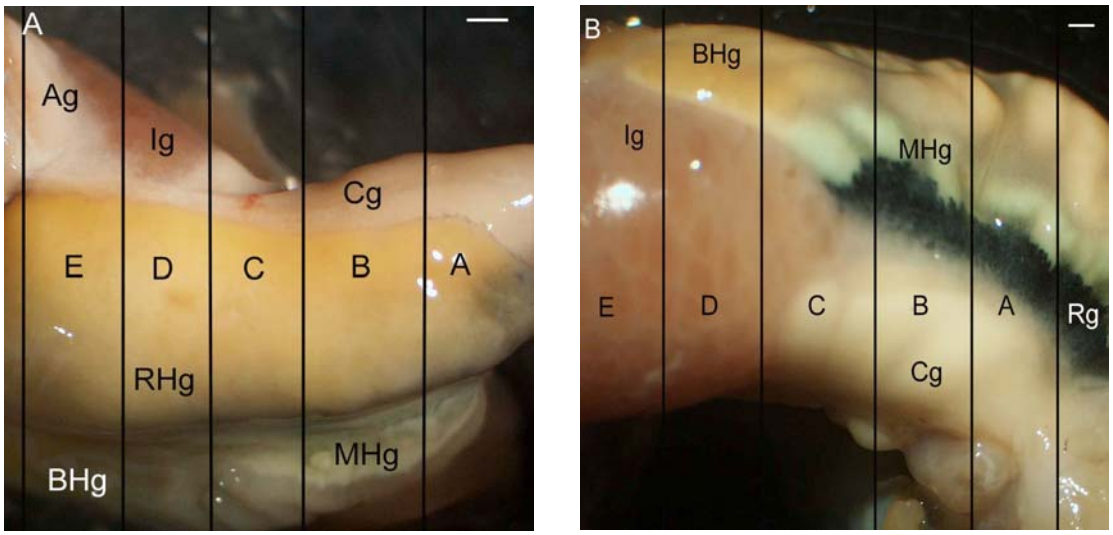


Figure 3. (A) Ventral and (B) dorsal views of the hypobranchial gland and pallial gonoduct of *D. orbita* showing from anterior to posterior, the five regions (A-E) where serial transverse sections were obtained. Ag, albumen gland; BHg, branchial hypobranchial gland; Cg, capsule gland; Ig, ingesting gland; MHg, medial hypobranchial gland; RHg, rectal hypobranchial gland. Scale bars = 1mm.

Triplicate paraffin sections were stained in 1) Modified Harris Haematoxylin and Eosin Y with Phloxine B (Thompson, 1966); 2) Toluidine Blue (Kramer and Windrum, 1954) and 3) Periodic Acid Schiff (McManus, 1946). These stains were chosen to allow comparison with previously defined hypobranchial secretory cell types (Chapter 3) and to assist with morphological descriptions where counterstaining

was detrimental to enzyme localization. Triplicate sections were also stained with the p-DMAB-nitrite method for tryptophan (Adams, 1957) and counter stained in Nuclear Fast Red (C.I. 60760).

Triplicate cryostat sections from each region (Fig. 3) were stained with an optimized bromoperhnol-red method for bromoperoxidase (Appendix II), an acid-hydrolysis method developed for tyrindoxyl sulphate (Appendix III) and the post-coupling method for arylsulphatase (Rutenburg et al., 1952). The bromophenol-red method is a histochemical technique adapted from Krenn et al. (1989) and Wever et al. (1991) based on the enzymatic conversion of phenol red (50 µM) to bromophenol blue in the presence of hydrogen peroxide (20µM) and potassium bromide (100Mm) (Fig. 4). The acid-hydrolysis is novel staining reaction based on a thin layer chromatography (TLC) technique by Baker and Duke (1976). This histochemical procedure employs HCl incubation as an alternative to arylsulphatase, to promote prochromogen hydrolysis and subsequent Tyrian purple genesis (Fig. 1). At high prochromogen concentrations, the resulting indole pigments form pink polymers, while at low concentrations, monomers and dimers appear blue-purple (Appendix III). All sections were examined under a compound light microscope (Olympus, BH-2).

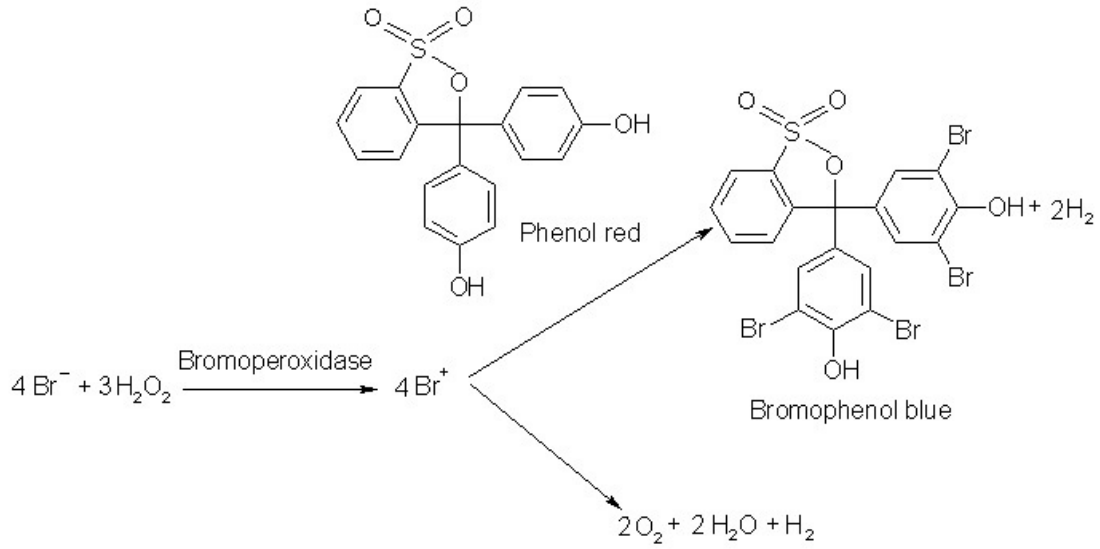


Figure 4. The bromoperoxidase-assisted conversion of phenol red to bromophenol blue proposed for the bromophenol-red method for bromoperoxidase. This reaction scheme was constructed in accordance with material presented by Butler and Walker, (1993) and Sels et al. (1999).

5. 3 Results

The ventral mantle of *D. orbita* is characterized by a hypobranchial gland, which extends from the ctenidium on the left to surround the ventral surface of the pallial gonoduct on the right (Fig. 3a). A vascular sinus separates the hypobranchial gland from the adjacent gonoduct and contains a prominent rectal gland (Fig. 3b) and the rectum. The hypobranchial gland is divided into three distinct regions, a left lateral branchial region, a medial depression, and a right lateral rectal region (Fig. 5). Upon dissection, the initially cream coloured secretion within the medial epithelium (Fig. 5) rapidly developed yellow, red, green and finally purple pigmentation, along

with a pungent sulphurous odour. The lateral regions were generally devoid of such secretion, although traces were observed on occasion (Fig. 5).

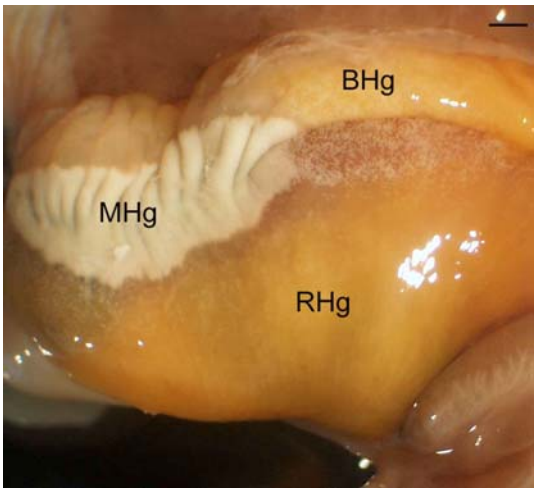


Figure 5. The ventral surface of the hypobranchial epithelium of *D. orbita.* Copious amounts of cream-coloured secretion were observed in the medial depression (MHg) and traces on the lateral branchial (BHg) and rectal (RHg) regions. Scale bar = 1mm.

Tryptophan was localized within hypobranchial (Fig. 6a) and rectum (Fig. 6b) epithelial cells, and vascular spaces of the dorsal sinus. Eosinophilic spherules of hypobranchial secretory cells common to the distal rectal and branchial epithelium stain strongly for tryptophan (Fig. 6a). The frequency of these cells decreased towards the medial region, which correlated with an increase in orthochromatic Toluidine Blue staining intensity. Columnar epithelial cells of the rectum contained two types of cytoplasmic spherules. One was weakly eosinophilic and orthochromatic with Toluidine Blue, while the second was strongly eosinophilic and tryptophan-positive (Fig. 6b). Spherules containing tryptophan were observed to coalesce with the basal membrane of epithelial cells in some individuals. An extracellular tryptophan-positive material of homogeneous composition was also observed

amongst basal and subepithelial muscle cells of the rectum (Fig. 6b) and hypobranchial gland (Fig. 6c), and within the dorsal vascular sinus. Tryptophan-positive and brown endogenous particles suspended in orthochromatic secretions invariably comprised rectum waste material. Tryptophan staining intensity and distribution from anterior to posterior was constant within all reproductive phases.

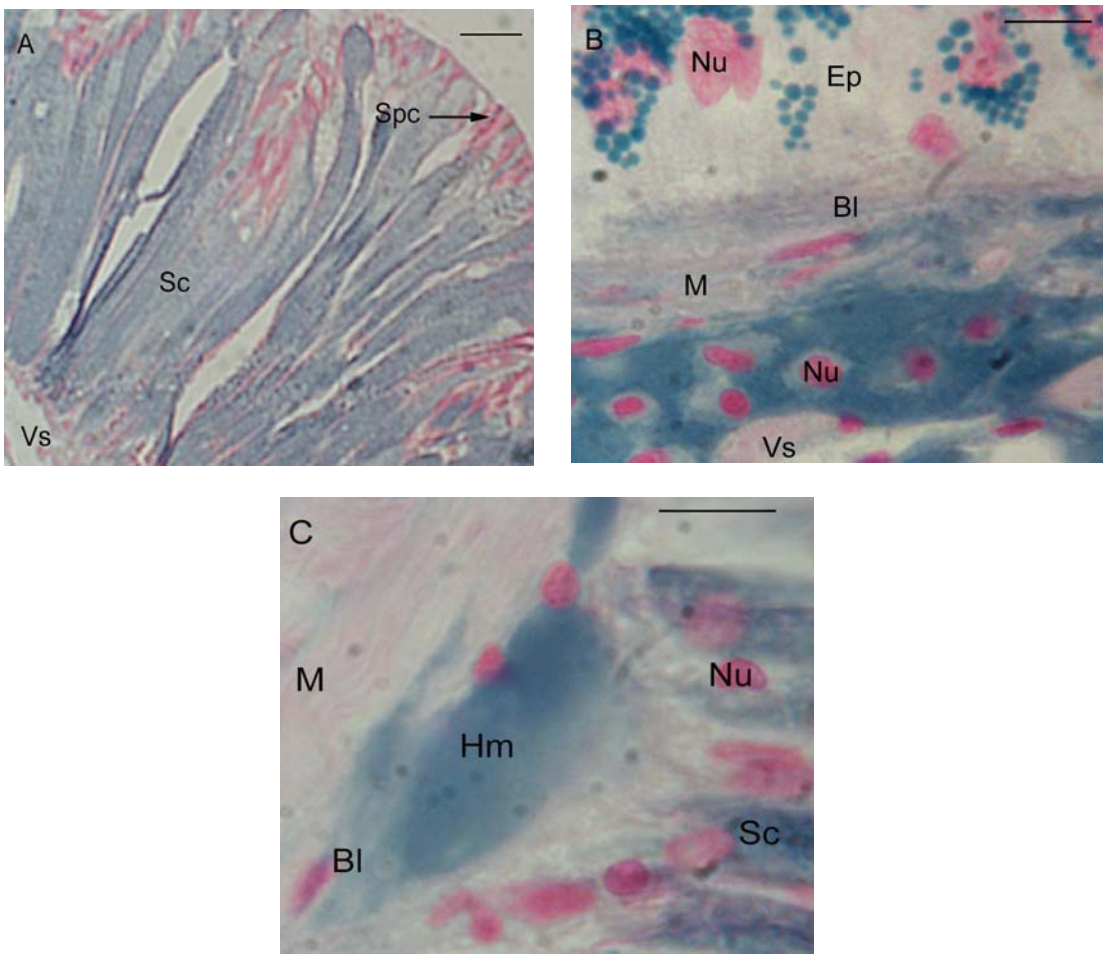


Figure 6. Demonstration of tryptophan (blue) within (A) hypobranchial secretory cells (Sc) of the distal branchial epithelium; (B) cytoplasmic spherules of rectum epithelial cells, the interstitial basal lamina (Bl) and subepithelial muscle (M), and vascular spaces (Vs) of the rectum; and (C) the lateral hypobranchial epithelium. Ep, epithelium; Hm, homogeneous material; Nu, nucleus; Spc, ciliated supportive cells. Scale bars = 50µm (A), 10µm (B-C).

The bromo-phenol red staining reaction identified sites of bromoperoxidase activity within the vascular sinus, hypobranchial epithelium and rectal gland of *D. orbita*. Intermittent deposits of bromophenol blue were observed within the apical (Fig. 7a) and basal region of lateral hypobranchial epithelial cells in all specimens. Apical mucous on the surface of these cells also stained for bromoperoxidase. Troughs formed by transverse folds in the epithelium stained more intensely than adjacent ridges in the majority of specimens (Fig. 7a). Ciliated supportive cells were visibly more abundant within these invaginations (Fig. 7b) due to higher epithelial cell concentrations. Weak cytoplasmic staining was observed on two occasions within metachromatic branchial hypobranchial cells and unstained medial cells. Staining intensity did not vary between anterior, medial and posterior hypobranchial sections or reproductive phases. A strong positive reaction for bromoperoxidase was also observed within subepithelial vascular spaces, interstitial basal cells of the medial hypobranchial gland, and the lumina of rectal gland acini in egg-laying specimens (Fig. 7c). Bromoperoxidase activity was also occasionally detected within the subepithelial vascular sinus of the branchial and rectal hypobranchial epithelium.

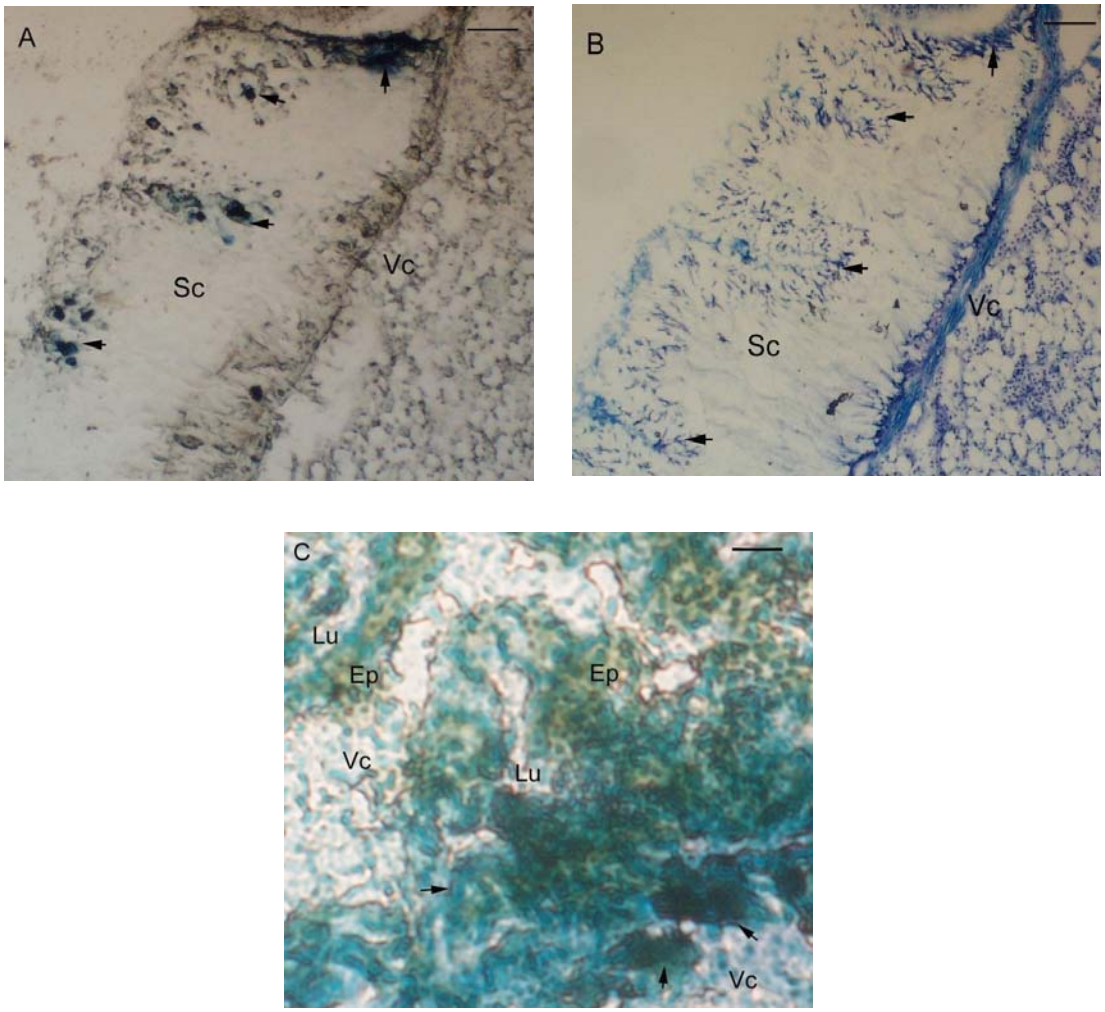


Figure 7. Bromoperoxidase staining in the apical region of ciliated supportive cells in the rectal hypobranchial epithelium, and within and surrounding rectal gland acini in *D. orbita*. (A) Bromophenol blue staining (arrows) within troughs of longitudinal folds in the hypobranchial epithelium, (B) high concentrations of ciliated supportive cells (arrows) within these invaginations stained in Toluidine Blue and (C) strong bromophenol blue staining (arrows) within vascular spaces (Vc) between the rectal gland and medial hypobranchial epithelium and moderate staining within rectal gland acini lumina (Lu) in egg-laying females. Ep, epithelium; Sc, secretory cells. Scale bars = 100µm (A-B) and 10µm (C).

The acid-hydrolysis method indicated tyrindoxyl sulphate presence within the hypobranchial gland and dorsal vascular sinus. Although secretions within medial hypobranchial epithelial cells failed to react with morphological staining procedures, acid-hydrolysis produced dark pink crystals throughout the cytoplasm (Fig. 8a) and apical mucous secretions of these cells within all specimens. Crystals were also present within the vascular sinus dorsal to the medial epithelium, surrounding ventral rectal gland acini (Fig. 8b) and occasionally within subepithelial spaces of the branchial hypobranchial region (Fig. 8c). Crystals were observed entangled in apical cilia of the distal branchial epithelium in five, and the rectal cilia of four specimens (Fig. 8d). In two of these latter cases, crystals were suspended within a homogeneous secretion which developed pale pink pigmentation (Fig. 8d). The majority of these individuals were in a post- or pre-reproductive state, although apical crystals were also evident in one copulating female. The cytoplasm of lateral hypobranchial epithelial cells failed to react with the acid-hydrolysis method in the majority of specimens (Fig. 8a). However in the case of two copulating females, tryptophan-positive cells of the branchial and rectal epithelium stained pale pink.

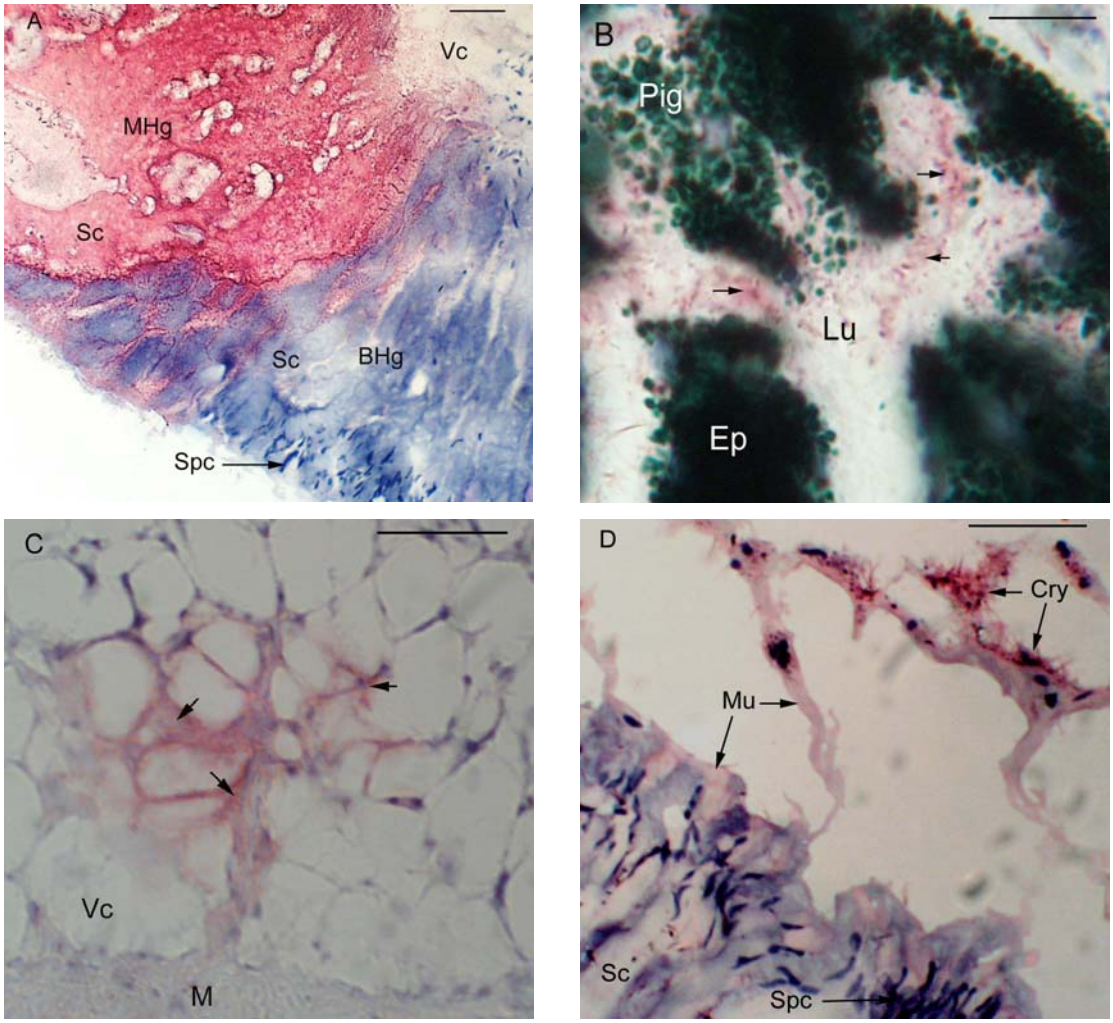


Figure 8. Tyrindoxyl sulphate localization within the hypobranchial gland, vascular sinus and rectal gland of *D. orbita*. (A) Medial hypobranchial (MHg) secretory cells (Sc) showing pink crystalline staining after application of the acid-hydrolysis method and negative lateral secretory cells (BHg) stained in haematoxylin. (B) Pink crystals (arrow) within the lumen (Lu) of rectal gland acini, (C) the subepithelial vascular sinus (arrows) of the branchial hypobranchial region; and (D) apical mucous secretions (Mu) of rectal hypobranchial epithelium. Cry, crystals; Ep, epithelial cells; M, muscle; Pig, endogenous pigments; Sc, secretory cells; Spc, ciliated supportive cells; Vs, vascular spaces. Scale bars = 50µm (A, C-D) and 10µm (B).

Arylsulphatase was invariably detected within the hypobranchial gland, rectum and vascular sinus of *D. orbita*. Enzyme distribution appeared consistent from anterior to posterior across all reproductive phases. Cytoplasmic products within all hypobranchial secretory cells remained unstained with the post-coupling method for arylsulphatase. Deposition of blue-purple azo dye was observed in the basal region of branchial (Fig. 9a), rectal and medial hypobranchial epithelial cells, the apical domain of branchial epithelial cells and the mucous entangled within cilia (Fig. 9a). In contrast, rectal cell apices stained red in the majority of egg-laying and post-reproductive females, and remained unstained in pre-reproductive and two copulating females. A feint line of purple staining was also produced between many secretory cells of the branchial and rectal hypobranchial gland, and occasionally between medial secretory cells. Within sections stained for morphological comparison, these regions correlate with the distribution of supportive cells, which rapidly taper from the epithelial surface and basal lamina due to constriction by adjacent secretory cells. Red staining was characteristic of all positive hypobranchial regions in pre-reproductive females.

Spherules within rectum epithelial cells (Fig. 9b) of all specimens and particles comprising waste occupying the lumen displayed intense purple staining after application of the post-coupling method for arylsulphatase. Homogeneous material within vascular spaces dorsal to the rectal, branchial (Fig. 9a) and medial hypobranchial gland also produced a weak purple stain. Granules in the lumina of rectal gland acini of five females from various reproductive phases also gained purple pigmentation. In the case of two egg-laying females, cytoplasmic granules stained bright blue (Fig. 9c).

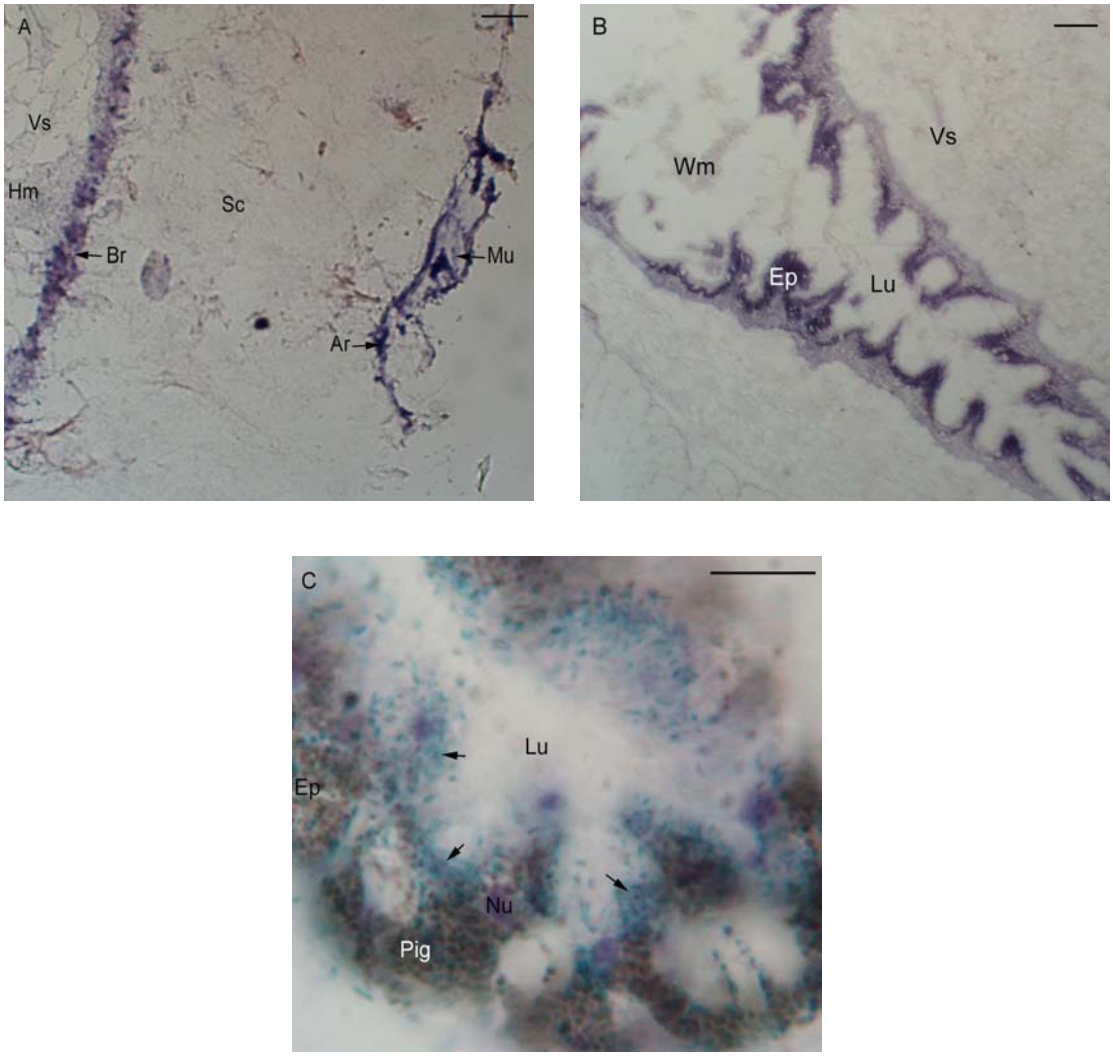


Figure 9. Localization of arylsulphatase by the post-coupling method within the rectum, hypobranchial and rectal glands. (A) High activity arylsulphatase within the apical (Ar) and basal region (Br) of the branchial hypobranchial epithelium and homogeneous material (Hm) within subepithelial vascular spaces (Vs), (B) the cytoplasmic contents of rectum epithelial cells (Ep), and (C) the lumen (Lu) of rectal gland acini. Mu, mucous; Nu, nucleus; Pig, endogenous pigments; Sc, secretory cells; Wm, waste material. Scale bars = 100µm (A-B), 10µm (C).

5.4 Discussion

Application of novel histochemical techniques for the demonstration of tyrindoxyl sulphate and bromoperoxidase in conjunction with standard histological methods has provided insight into the biosynthetic origin and regulation Tyrian purple genesis. Two sites of prochromogen synthesis appear possible within the hypobranchial gland of *D. orbita*. Endocytosed tryptophan-positive material sourced from rectum waste appears to be stored within secretory cells of the branchial and rectal hypobranchial epithelium, which upon liberation onto the epithelial surface, is united with bromoperoxidase to form crystals of tyrindoxyl sulphate (Fig. 10). Prochromogen synthesis also occurs in the vascular sinus immediately dorsal to the medial hypobranchial gland for storage and secretion by medial epithelial cells (Fig. 10). It is proposed that hydrolysis of tyrindoxyl sulphate is regulated, and only occurs once arylsulphatase is exocytosed onto the surface by adjacent supportive cells (Fig. 10). Evidence for the controlled genesis of bioactive intermediate precursors and choline esters within the hypobranchial gland of *D. orbita* strongly suggests an ecological function for Tyrian purple synthesis within the Muricidae.

Histochemical evidence suggests that tyrindoxyl sulphate is synthesized from dietary derived tryptophan. Waste material containing tryptophan-positive particles invariably occupied the rectum of *D. orbita*. As tryptophan biosynthesis is almost completely restricted to plants (Crawford, 1989; Bentley, 1990; Hermann et al., 1992), it is presumed this amino acid originates from a diet of herbivorous prey. The presence of tryptophan-positive cytoplasmic spherules within specific rectal epithelial

cells implies that dietary tryptophan is endocytosed and concentrated by these cells (Fig. 10).

The distribution of tryptophan-positive material suggests this amino acid is transported from the rectum to the vascular sinus for endocytosis by the hypobranchial epithelial cells (Fig. 10). Homogeneous tryptophan-positive material was observed within extracellular spaces of the rectum basal lamina, subepithelial muscle and the vascular sinus adjoining the hypobranchial gland. Although molluscan haemocyanin contains tryptophan residues (Waxman, 1975; Avissar et al., 1986), localization in the vascular sinus is not likely due to haemocyanin as the N-terminal amino acid of muricid haemocyanin is serine (Idakieva et al., 1993), which fails to form a blue pigment with p-DMAB-nitrite (Adams, 1957). However, haemocyanin may function as a transport protein through the temporary condensation of tryptophan and serine, thereby resulting in p-DMAB-nitrite staining. Alternatively, free tryptophan may be transported in the haemolymph plasma. Tryptophan-positive material was also observed amongst basal cells of hypobranchial secretory cells containing tryptophan-positive spherules (Fig. 6c). These cells dominate the distal lateral epithelium (Fig. 6a), but reduce in frequency towards the medial epithelium. Correlations in biochemistry and distribution indicate that these cells are those previously thought be involved in the sequestration of tryptophan in *D. orbita* (Chapter 3). Thus, it appears that dietary tryptophan is mobilized in the haemolymph, accumulated by these prominent hypobranchial secretory cells and stored as a membrane-bound mucopolysaccharide-protein complex until exocytosis (Fig. 10).

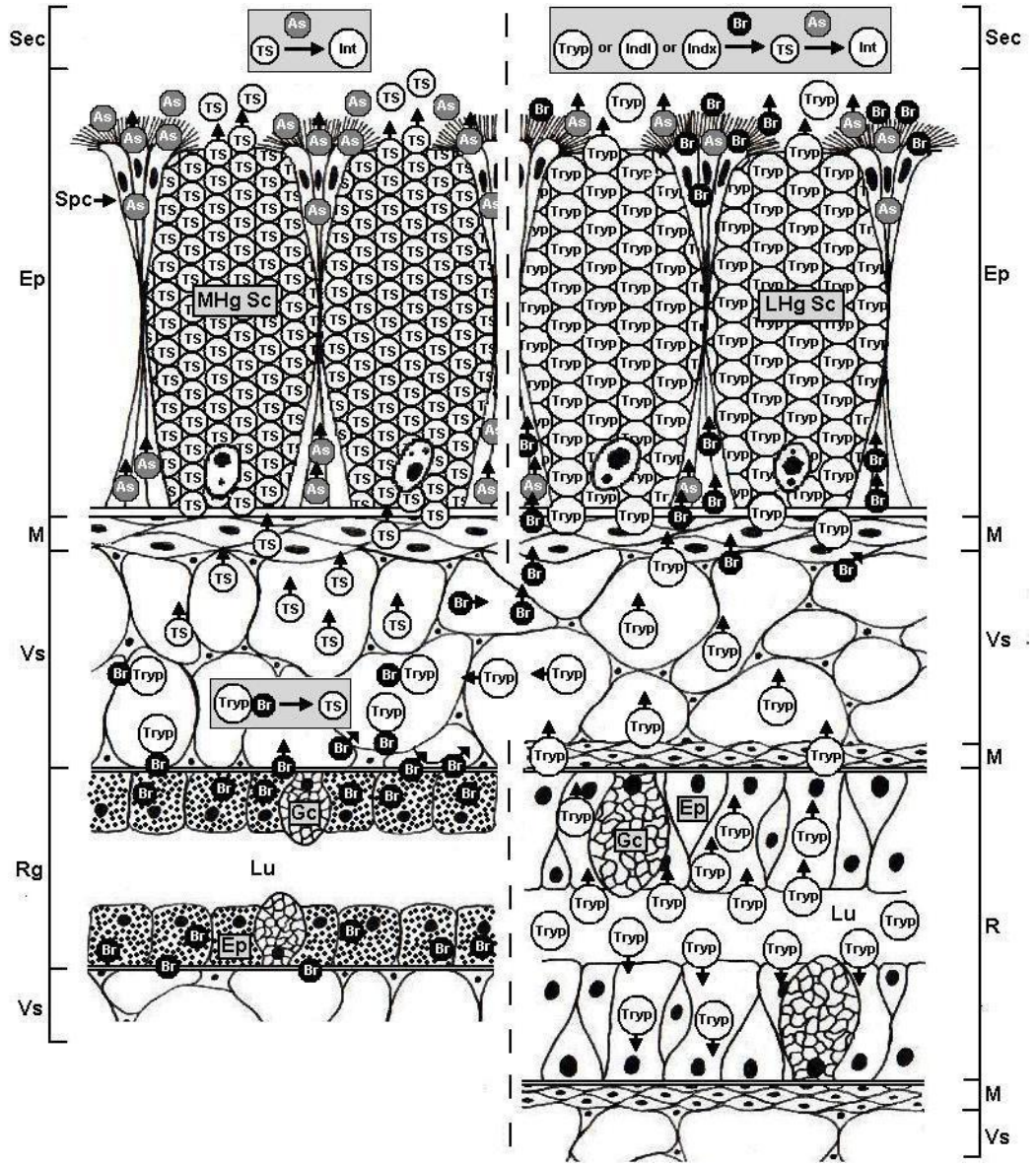


Figure 10. Constitutive tyrindoxyl sulphate (TS) biosynthesis in the subepithelial vascular sinus (Vs) of medial hypobranchial secretory cells (MHg Sc), and induced synthesis within secretions (Sec) of lateral cells (LHg Sc). Tryptophan (Tryp) is endocytosed by rectum (R) epithelial cells (Ep) and transferred via the vascular sinus (Vs) to the lateral epithelium. Bromoperoxidase (Br) in the rectal gland (Rg) is also mobilized in the vascular sinus. Intermediate precursor (Int) genesis occurs on the epithelial surface upon liberation of arylsulphatase from ciliated supportive cells (Spc). Gc, goblet cells; Lu, lumen; M, muscle. This schematic is not to scale.

Tryptophan-containing cells have been identified in hypobranchial glands of the muricids *M. brandaris* (Bolognani-Fantin and Ottaviani, 1981), *M. granulata* (Srilakshmi, 1991) and *P. pansa* (Naegel and Aguilar-Cruz, 2006). As tryptophan is the indole precursor, detection of this amino acid has been previously assumed to double for localization of Tyrian purple precursors (Naegel and Aguilar-Cruz, 2006). However, as indole precursors (Fig. 1) lack the functional group to form the ß-carboline derivative (Fig. 2) in the histochemical techniques applied (Adams, 1957; Davenport, 1960), simultaneous demonstration is unlikely. Application of the p-DMAB-nitrite method (Adams, 1957) in conjunction with the acid-hydrolysis technique in this investigation highlights the error of this previous assumption. Production of dark pink crystals after acid-hydrolysis identified secretory cells of the medial hypobranchial epithelium as the primary region of tyrindoxyl sulphate storage (Fig. 8a). The absence of tryptophan within these cells coupled with detection of this amino acid in morphologically distinct lateral secretory cells (Fig. 6a), indicates that the prochromogen and precursor are stored separately in *D. orbita* (Fig. 10).

The distribution of these tyrindoxyl sulphate-containing cells in *D. orbita* correlates well with the findings of Erspamer (1946), which indicate prochromogen presence exclusively within the medial hypobranchial gland of *Murex trunculus* and *M. brandaris*. Cells, termed “clear” or “empty” due a lack of cytoplasmic staining, have been reported in all muricid hypobranchial glands studied to date (Bolognani-Fantin and Ottaviani, 1981; Srilakshmi, 1991; Roller et al., 1995; Naegel and Aguilar-Cruz, 2006). Similarly, the medial cells of *D. orbita* failed to stain with any of the morphological stains applied (Chapter 3). The apparent lack of proteins and carbohydrates within these cells, coupled with the crystalline morphology of dye

pigments after acid-hydrolysis implies that the prochromogen, an indoxyl sulphate salt, may be present in a pure state. Furthermore, the apparent absence of these cells in non-muricid species documented to date (Tarao, 1935; Ronkin, 1952; Hunt, 1973; Ottaviani, 1978) suggests that the "empty" cells of other muricids also contain Tyrian purple prochromogens.

Histochemical evidence suggests that two mechanisms of prochromogen synthesis occur within the hypobranchial gland of *D. orbita*. In addition to the cytoplasm of medial epithelial cells, crystals of tyrindoxyl sulphate were detected within the vascular sinus dorsal to these cells and in apical secretions of the rectal (Fig. 8d) and branchial epithelium. The invariable presence of crystals adjacent to the proposed site of prochromogen storage in medial hypobranchial epithelial cells suggests that synthesis occurs within these vascular spaces prior to epithelial sequestration (Fig. 10). The absence of crystals in the cytoplasm of lateral cells (Fig. 8a) indicates that tyrindoxyl sulphate is also synthesized on the epithelial surface upon exocytosis of tryptophan (Fig. 10).

The genesis of an unusual pale pink dye product after acid-hydrolysis suggest that tryptophan is converted to indole or indoxyl within the cytoplasm prior to prochromogen synthesis on the epithelial surface (Fig. 10). On two occasions tryptophan-positive cells of the lateral hypobranchial epithelium gained pale pink pigmentation after acid-hydrolysis, while in another two individuals, apical crystals were suspended within a homogeneous substance which also stained pale pink (Fig. 8d). During method development, trials indicated that tryptophan fails to produce a coloured product after exposure of HCl (Appendix III). Nevertheless, it is possible that prior to bromoindoxyl sulphate synthesis on the surface, tryptophan degradation

to indole or indoxyl occurs within these lateral cells, generating intermediates which stain for tryptophan and have the potential to develop pink pigmentation. Of these intermediates, 3-indoleacetic acid is known to react with the p-DMAB nitrite method (Adams, 1957) and may be converted via indoxyl to pink indirubin (see Allegri et al., 2006). The infrequent occurrence of pale pink staining further suggests that indoxyl synthesis from tryptophan within these cells is regulated. Although cytoplasmic indoxyl synthesis may theoretically explain the staining of some lateral cells and apical secretions by both acid-hydrolysis and the p-DMAB nitrite method, the location of enzymes involved in indole synthesis and the influence of HCl on indoxyl intermediates requires further investigation.

Bromination of tryptophan, indole, 3-indoleacetic acid or indoxyl is required for the genesis of tyrindoxyl sulphate. Application of the bromophenol-red method identified comparatively high concentrations of bromoperoxidase amongst rectal gland acini (Fig. 7c), subepithelial vascular spaces and basal cells of the medial hypobranchial epithelium where tyrindoxyl sulphate is most abundant (Fig. 10). In *D. orbita*, this conspicuous rectal gland commences and terminates with the medial hypobranchial epithelium (Fig. 3b), which suggests it may be associated with dye synthesis. Bacteria are known to produce bromoperoxidase (Butler and Walker, 1993; Gribble, 1999) and synthesize brominated natural products (Garson, 1993). Thus, it is possible that bromoperoxidase is acquired from symbiotic bacteria for precursor bromination.

Bacteria have been observed within specialized invaginations of the rectal gland epidermis in the muricid *Nucella lapillus* (Andrews, 1992). As the rectal gland functions in the catabolism of haemolymph macromolecules (Andrews, 1992), this

gland is an ideal environment for the bacterial synthesis of bromoperoxidase. Bromoperoxidase contains tryptophan residues among other amino acids (Wever et al., 1988), which would be made readily available during degradation of haemolymph proteins such as haemocyanin. Furthermore, active transport is known to occur across the rectal gland basal lamina (Andrews, 1992). Thus, it is possible that bromoperoxidase is synthesized by symbiotic bacteria, released into the vascular sinus and mobilized by the haemolymph for bromoindoxyl sulphate synthesis (Fig. 10). Twelve neogastropod families possess a rectal gland; the Muricidae, Conidae, Volutidae, Olividae, Mitridae, Columbariidae, Vexillidae, Volutomitridae, Marginellidae, Turbinellidae, Cancellariidae and Terebridae (see Ponder, 1973; Taylor and Morris, 1988). Of these, species of the Muricidae and Conidae synthesize metabolites which contain tryptophan residues brominated in the 6-position (Jimenez et al., 1997; Westley et al. 2006). Furthermore, chromatic reactions have been observed in hypobranchial secretions of the Volutidae (Weaver and Du Port, 1970), Olividae (Marcus and Marcus, 1959) and Mitridae (Schimelman, 1982). Thus, it would be of interest to investigate such correlations in the remaining families, as the rectal gland may be of greater functional significance than previously thought.

It should be noted that bromoperoxidase activity was only detected within rectal gland lumina of egg-laying females, which may by due to an increased demand for Tyrian purple precursors during capsule manufacture. Bioactive precursors occur in muricid egg masses, where they are thought to function in the chemical defence of encapsulated larvae (Benkendorff et al., 2000, 2001, 2004). It has been proposed that precursors are incorporated into egg masses as a form of maternal investment (Westley et al., 2006). If the rectal gland is the source of bromoperoxidase in the

Muricidae, activity or enzyme concentration may become heightened within egg-laying females to provide for the incorporation of bioactive intermediate precursors into egg masses.

Bromoperoxidase activity was also detected within the basal and apical region of lateral hypobranchial secretory cells. In comparison to adjacent ridges, staining was more intense in troughs of transverse epithelial folds where ciliated supportive cells occur in high concentration. Although intracellular localization was not possible, the close correlation of staining with ciliated supportive cell distribution (Fig. 7a-b) suggests they secrete bromoperoxidase onto the epithelium surface (Fig. 10). Ciliated cells between groups of secretory cells appear common to buccinid and muricid hypobranchial glands (Hunt, 1973; Bolognani-Fantin and Ottoviani, 1981; Srilakshimi, 1991; Roller et al., 1995). These cells are thought to function in providing support, combining secretions released from adjacent cells, and moving secretory products across the epithelial surface (Hunt, 1973; Bolognani-Fantin and Ottaviani, 1981; Roller et al., 1993). Although cytoplasmic secretory granules are not visible by light microscopy, ultrastructural investigation has previously revealed the presence of vacuoles containing structures resembling cytosomes, lysosomes, microtubules and filaments (Hunt, 1973). It is possible that enzymes such as bromoperoxidase could be stored in these vacuoles and transported to the surface with the assistance of microtubules. The intermittent nature of apical and basal bromophenol staining indicates that bromoperoxidase was activated in the process of liberation, or in comparatively high concentration in some cells, but not all supportive cells, at the time of sectioning. This suggests that enzyme release or activation is regulated, which may be a mechanism controlling prochromogen bromination.

Once secreted, bromoperoxidase may be combined with tryptophan, indole 3-indoleacetic acid or indoxyl from adjacent secretory cells through ciliary action to facilitate precursor bromination (Fig. 10). Like all haloperoxidases, bromination by bromoperoxidase requires hydrogen peroxide (Fig. 4). There are several potential sources of hydrogen peroxide, the most obvious being seawater where it is produced photochemically from dissolved organic matter (Miller et al., 2005). Seawater introduced to the mantle cavity is in constant contact with the hypobranchial epithelium, which would theoretically unite dissolved hydrogen peroxide with liberated bromoperoxidase to promote precursor bromination. Oxidative enzymes may also induce hydrogen peroxide production on the epithelial surface or within peroxisomes. Peroxisomes are ubiquitous organelles, which contain up to 60 different enzymes and function in fatty acid metabolism (Cancio et al., 2000). Of these enzymes, peroxisomal oxidases catalyze the oxidation of various organic substrates, producing hydrogen peroxide (Fahl et al., 1984). As peroxisomes are part of the secretory pathway, supportive cells may release either hydrogen peroxide or oxidase onto the hypobranchial epithelium in conjunction with bromoperoxidase for the bromination of precursors liberated by adjacent secretory cells.

In addition to bromoperoxidase, ciliated supportive cells also appear to secrete arylsulphatase (Fig. 10). Morphological comparisons indicate that arylsulphatase staining correlates with the visible distribution of supportive cells, which rapidly taper from the epithelial surface and basal lamina due to constriction by adjacent secretory cells. The presence of this enzyme within these cells is also supported by the genesis of Tyrian purple along the apical and basal region of medial and some rectal hypobranchial cells in sections exposed to sunlight during trials for the acid-

hydrolysis hydrolysis method (Appendix III). This mechanism of enzyme delivery would restrict prochromogen hydrolysis to the surface of the hypobranchial epithelium, and ultimately regulate the genesis of bioactive intermediates to Tyrian purple.

Arylsulphatase activity varied with hypobranchial region and reproductive status. Enzyme activity was consistently low within all pre-reproductive females. This would theoretically result in reduced prochromogen hydrolysis and subsequent Tyrian purple synthesis, which correlates well with previous observations of decreased hypobranchial secretory activity outside the reproductive season (Fretter and Graham 1994). Enzyme activity was generally high within the basal region of supportive cells, but low in the apical domain of those in the rectal region of some egg-laying and post-reproductive females, and absent in two copulating females. Reductions in the arylsulphatase activity of supportive cells in the rectal hypobranchial epithelium may be related to differences in enzyme packaging or specificity. Six (A-F) substrate-specific arylsulphatases occur in mammalian tissues (Tobacman, 2003). Of these, arylsulphatase B is an acid hydrolase which functions in the hydrolysis of sulphated polysaccharides (Bhattacharyya et al., 2007). As cells containing sulphated acid mucopolysaccharide are characteristic of the branchial hypobranchial epithelium of *D. orbita* (Chapter 3), an enzyme similar to arylsulphatase B may be associated with this region. Furthermore, liberated sulphate (SO_4^{-2}) or the reduction of sulphuric acid (H_2SO_4), may then be utilized in the synthesis of the indoxyl sulphate prochromogen (e.g. **1**). The post-coupling method applied in this investigation (Rutenburg et al., 1952) is not specific to arylsulphatase class. Consequently, it is possible that the enzyme required for prochromogen

hydrolysis is of consistently low activity, but appears high in the branchial region due to staining of a high activity arylsulphatase for polysaccharide desulphation. Alternatively, arylsulphatase is known to possess high activity when present in the endoplasmic reticulum where synthesis occurs, but low activity once packaged into lysosomes for transport (Bhattacharyya et al., 2007). Consequently, variations in arylsulphatase activity may depend on the stage of enzyme synthesis. These reasons may also explain failure to detect arylsulphatase outside of the anterior medial hypobranchial gland of *Murex trunculus* and *M. brandaris* (Erspamer, 1946).

Cytoplasmic spherules in rectum epithelial cells (Fig. 9b), granules within lumina of rectal gland acini (Fig. 9c) and sinus plasma, also demonstrated various levels of arylsulphatase activity. Arylsulphatase within these regions is most likely associated with normal cellular metabolism and cell mediated immune responses, as lysosomal arylsulphatases are involved in macromolecule catabolism (Tobacman, 2003) and pathogen degradation (Wootton and Pipe, 2003).

Coincidence of tryptophan-positive material, bromoperoxidase and tyrindoxyl sulphate suggests there are two possible sites where prochromogen synthesis occurs. The most active is within the vascular sinus immediately dorsal of the medial hypobranchial epithelium (Fig. 10). Once synthesized, crystals of bromoindoxyl sulphate appear to be concentrated by medial epithelial cells for secretion onto the epithelial surface (Fig. 10). As both bromoperoxidase and a tryptophan-positive material occur in abundance within this region, regulation of prochromogen synthesis may depend on the availability of tryptophanase and decarboxylase for indole biosynthesis (see Allegri et al., 2006). Alternatively, medial secretions may be unregulated in a similar manner to the constitutive secretion of goblet cells, which

maintain the protective mucous coat of intestinal tissues (Ho and Shekels, 2003). The copious amount of secretion invariably observed within the medial surface depression and epithelial cells indicates that constant prochromogen synthesis and secretion is likely. To be metabolically viable, prochromogens must therefore be required to maintain a specific environment or function on the epithelial surface or mantle cavity, which is of significant selective benefit to the Muricidae.

In contrast, prochromogen synthesis on the lateral epithelial surface appears to be induced. A previous electron microscopy study of the hypobranchial surface in *Thais haemastoma* suggested that lateral secretions are directed towards the medial depression for storage prior to release (Roller et al., 1995). However, supplementing a region of constitutive secretion with biochemically identical secretions from another source appears to be an unusual strategy. Furthermore, declines in the frequency of tryptophan-positive cells towards the medial depression suggests the demand for regulated prochromogen synthesis also declines due to constitutive synthesis within this region. In general, controlled mucous synthesis occurs in response to certain chemical or mechanical stimuli, such as hormones, neurotransmitters, chemical and physical irritants (Ho and Shekels, 2003). Thus, it appears likely that Tyrian purple precursor synthesis and secretion is induced within the lateral hypobranchial epithelium of the Muricidae.

The significance of synthesizing precursors to bioactive metabolites in two distinct manners is unclear as the functional role of hypobranchial gland remains unresolved. Nevertheless, the constitutive and regulated synthesis of prochromogens in the medial and lateral hypobranchial epithelium, respectively, appears common to the Muricidae. Choline esters associated with prochromogens (Fig. 1) have been

isolated to the medial region (Vincent and Jullien, 1938; Erspamer and Dordoni, 1947l, Erspamer, 1948; Keyl et al., 1957; Andrews et al., 1991) or found to be 50 times more abundant in comparison to the lateral region (Erspamer, 1948; Roseghini and Fichman, 1973). Originally, it was thought that Tyrian purple precursors were dietary-derived and bioactive intermediates were a coincidence of detoxification (Verhecken, 1989). One agreed upon functional role of the hypobranchial gland involves the trapping and cementing of particles introduced by the inhalant stream for subsequent expulsion (Fretter and Graham, 1994). However, this role fails to explain the significance of Tyrian purple secondary metabolites and the division of secretory pathways. Another proposed function, which could theoretically be mediated by the regulated synthesis and hydrolysis of prochromogens in the lateral hypobranchial epithelium, is the use of physiologically-active choline esters in prey capture (Keyl et al., 1957; Whittaker, 1960; Roseghini et al., 1970; Huang and Mir, 1971; Ottaviani, 1978; Bolognani-Frantin and Ottaviani, 1981; Roller et al., 1984; Srilankshmi, 1991; Roseghini et al., 1996). However, this hypothesis neglects the fact that cytotoxic precursors, of no apparent use during prey capture, are simultaneously generated during prochromogen hydrolysis. Thus, it is clear that future investigation into the evolutionary significance of hypobranchial secondary metabolites should strongly consider the heterogeneous activity and mechanisms of synthesis associated with this intriguing biosynthetic pathway.

5. 5 Conclusion

Histochemical evidence for the regulated and constitutive *de novo* synthesis of prochromogens indicates that these natural products are of selective benefit to the

Muricidae. The findings presented here also suggest that the liberation of physiologically active choline esters and subsequent genesis of antimicrobial precursors by arylsulphatase requires induction. It is anticipated that the insight provided by this investigation into the biosynthetic origin and regulation of Tyrian purple prochromogen and precursor synthesis will aid future attempts to define the functional significance of this biosynthetic pathway and the gastropod hypobranchial gland. It is also hoped that the advantages of adopting a histochemical approach to natural product research will be considered in subsequent investigations aimed at elucidating the biosynthetic origin of marine natural products.

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