Appendix III

The development of a novel histochemical method for the demonstration of Tyrian purple tyrindoxyl sulphate in animal tissue sections

Histochemical methods for the demonstration of bromoindoxyl sulphate prochromogens to Tyrian purple are not currently available in the literature. Consequently, a novel technique was developed to determine potential sites of tyrindoxyl sulphate synthesis in the hypobranchial gland of *Dicathais orbita*.

3. 0 Specimen collection and maintenance

Nine female *D. orbita* were employed for the development of histochemical techniques (3 x extracts, 3 x formalin-fixed sections and 3 x cryostat sections). These specimens were sampled from a population of mature *D. orbita,* held at Flinders University Marine Aquarium, Adelaide, South Australia. Animals were maintained on a diet of *Turbo undulatus*, *Mytilus edulis planulatus,* juvenile *Haliotis* spp. and *Donax deltoides* within a subtidal recirculatory aquarium at 15°C, a salinity of ~35.5ppt and a 12/12h day/night cycle. Specimens comprising the captive population were collected from the rocky intertidal and subtidal regions of the Adelaide metropolitan coast, Fleurieu and Eyre peninsulas of South Australia.

3. 1 Dissections

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. Tissue was prepared for sectioning according to the specifications outlined below (section 3.3) and examined under a compound light microscope (Olympus, BH-2).

3. 2 Trial 1: HCl and enzyme prochromogen hydrolysis

Tyrian purple genesis occurs through a series of hydrolytic, oxidative and photolytic reactions (Fig. 1) (Cooksey, 2001). Within muricid hypobranchial glands, conversion of Tyrian purple prochromogens (e.g. **1**) to intermediate indole precursors (e.g. **2-4)** commences with prochromogen hydrolysis by the enzyme arylsulphatase (Fig. 1) (Cooksey, 2001). Spraying with 1M HCl and heating to 100°C has also been found to facilitate prochromogen hydrolysis (Fig. 1) within hypobranchial ethanol extracts after thin layer chromatography (TLC) (Baker and Duke, 1976). In both instances, subsequent exposure to oxygen and light results in the genesis of Tyrian

purple dye pigments (Fig. 1, e.g. **5**) (Baker & Sutherland, 1968; Baker and Duke, 1976; Cooksey, 2001). Consequently, these methods are potentially useful as templates for the development of a histochemical method for the demonstration of prochromogens, such as tyrindoxyl sulphate (**1**).

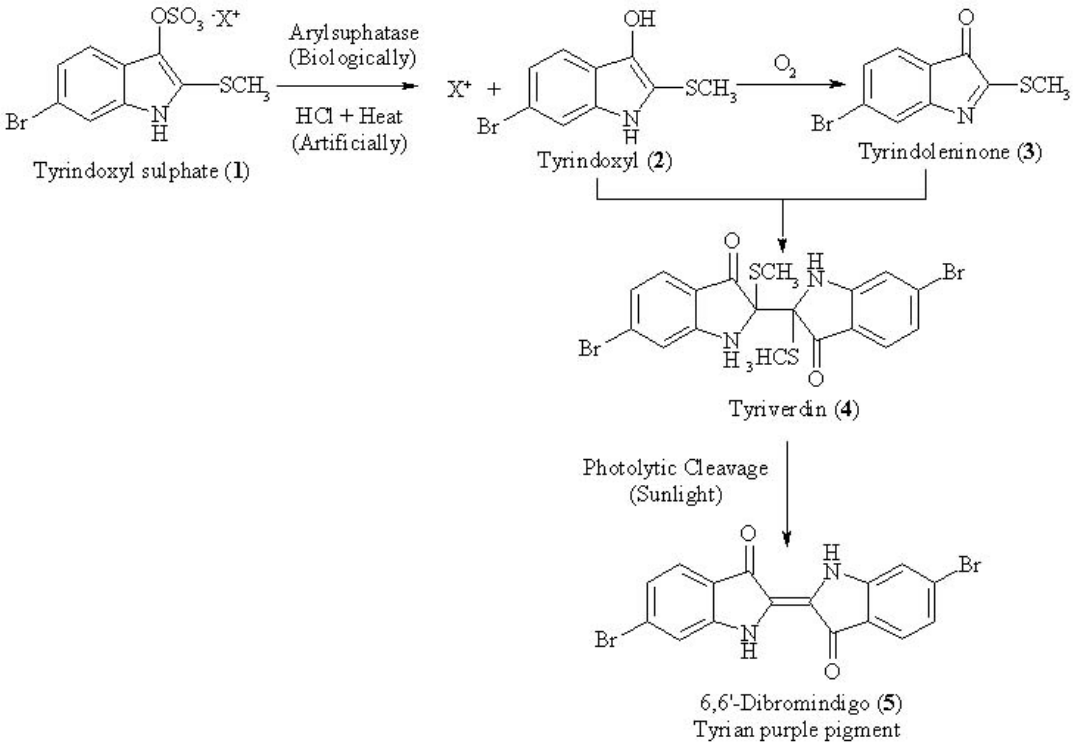

Figure 1. Genesis of Tyrian purple intermediate precursors and dye pigments from the prochromogen, tyrindoxyl sulphate in *D. orbita*. Biosynthetic pathway adapted from Baker and Duke (1976) and Cooksey (2001a).

The acid-hydrolysis visualization technique for Tyrian purple prochromogens (Baker and Duke, 1976) requires modification, as subjecting sections to 1M HCl immersion and high temperatures would be destructive to delicate tissues. It is hypothesized that concentrated HCl vapours and the heat generated by the liberation

of these vapours may be effective at hydrolyzing tyrindoxyl sulphate. Thus, this method of prochromogen visualization was trialed on hypobranchial ethanol extracts after TLC. Endogenous and commercial molluscan arylsulphatase were also trialed by TLC and the dye yields compared to those resulting from acid-hydrolysis. To ensure these proposed techniques discriminate between the indole precursor, tryptophan, and Tyrian purple prochromogens, the potential formation of dye pigments from L-tryptophan post arylsulphatase or HCl exposure was also investigated by TLC

3. 2. 1 Hypobranchial gland extract preparation and TLC procedure

Triplicate hypobranchial glands were extracted overnight in absolute ethanol and gravity filtered through glass wool. Extracts were separated on aluminum-backed silica gel plates (Merck), employing an n-butanol-EtOH-acetic acid-water (8:2:1:3) solvent system (Baker & Duke, 1976).

3. 2. 2 Enzyme and tryptophan preparation (TLC)

D. orbita arylsulphatase was precipitated out of aqueous extracts (Fig. 2) of hypobranchial gland residues remaining after ethanolic extraction according to Baker and Sutherland (1968). The enzyme concentrate was then pipetted into a clean amber vial. Molluscan arylsulphatase from *Helix pomatia* (Sigma, S9626) was prepared to a concentration of 5.0units/ml in 0.2% (w/v) sodium chloride solution according to the quality control enzymatic assay (Sigma, 1997). L-tryptophan (Sigma-Aldrich, T0254) was dissolved in ethanol to a concentration of 50mM.

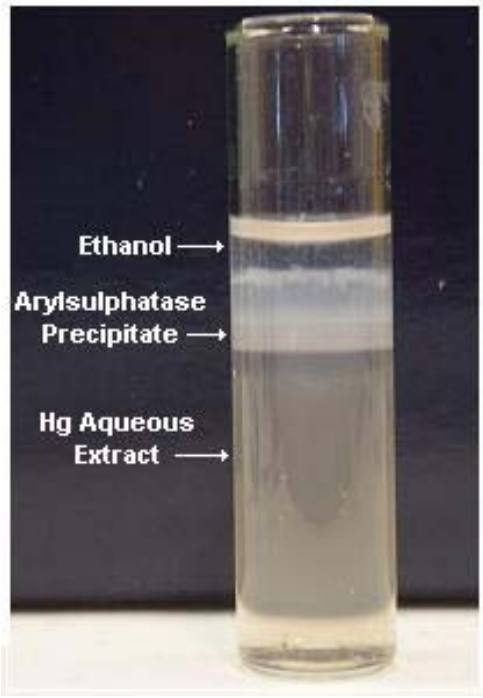

Figure 2. Precipitation of *D. orbita* arylsulphatase from aqueous hypobranchial gland (Hg) extracts.

3. 2. 3 Enzyme and HCl incubation procedures (TLC)

Arylsulphatase incubation

D. orbita and *H. pomatia* arylsulphatase solutions were applied by pipette to triplicate TLC plates of separated hypobranchial gland extracts and L-tryptophan. Sodium chloride solution (0.2%) or distilled water was applied by pipette to negative control plates. All plates were then developed in direct sunlight for 1h.

HCl incubation

Triplicate TLC plates of separated hypobranchial gland extracts and L-tryptophan were placed on a platform within a sealed chamber containing a small amount of concentrated (12M) HCl, exposed to the vapours for 5min and developed in direct sunlight for 1h. Negative control plates were developed in sunlight for 1h, without prior treatment.

3. 2. 4 Enzyme and acid incubation dye yield comparison (TLC)

Colourless UV-active spots (R_f 0.80) gained pale stippled purple pigmentation in the presence of *D. orbita* arylsulphatase concentrate (Fig 3a), while *H. pomatia* arylsulphatase failed to promote the genesis of dye pigments. During HCl exposure, UV-active spots at R_f 0.80 also developed intense pink pigmentation, which faded to a purple colour towards the base line as compound concentration decreased (Fig. 3b). Additional orange spots (R_f 0.95), which turned purple upon sunlight exposure, were also observed and are most likely the intermediate precursor tyrindoleninone (Benkendorff et al., 2000). Plates containing L-tryptophan failed to gain any pigmentation after enzyme or acid incubation. Plates developed in sunlight remained colourless or in the case of one replicate, stained light orange. Similarly, incubation in sodium chloride solution or distilled water (Fig. 3a) failed to induce a colorimetric reaction.

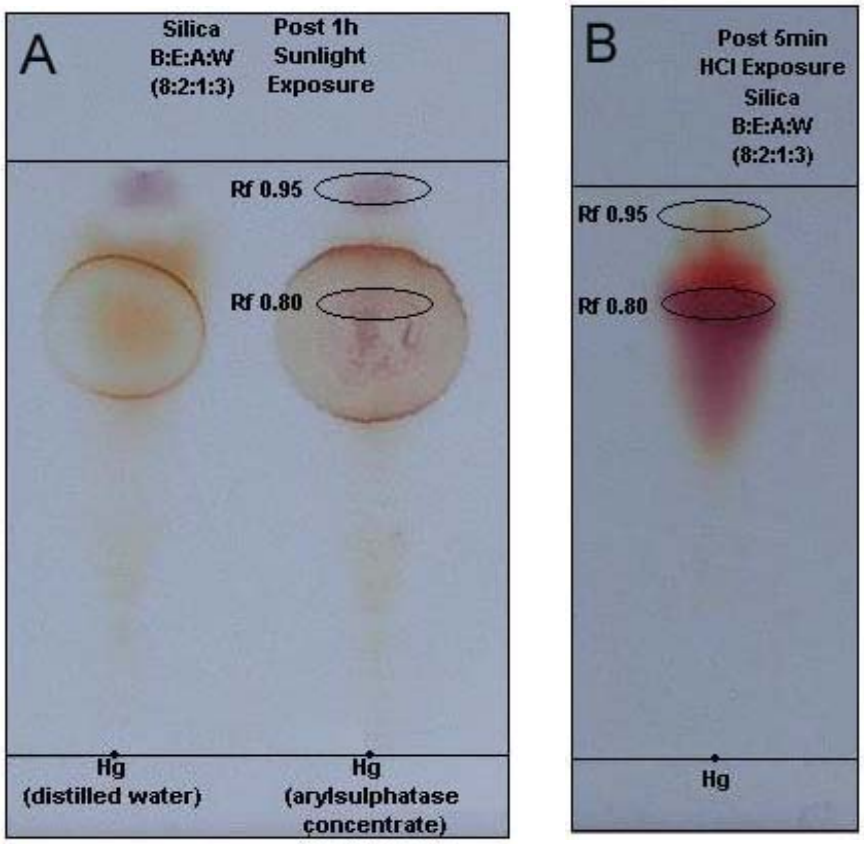

Figure 3. TLC plate of a hypobranchial gland (Hg) extract showing the formation of Tyrian purple pigments within spot R_f 0.80 after (A) *D. orbita* arylsulphatase and (B) HCl incubation.

These results indicate that although enzyme incubation in *D. orbita* arylsulphatase effectively hydrolyzes tyrindoxyl sulphate (Fig. 3a), the visible dye yield is poor in comparison to that catalyzed by acid-hydrolysis (Fig. 3b). Interestingly, *H. pomatia* arylsulphatase failed to hydrolyze tyrindoxyl sulphate, which suggests that muricid arylsulphatase may be substrate-specific in action. The negative result obtained after acid incubation of L-tryptophan plates, further implies

that the acid-hydrolysis method of prochromogen visualization effectively discriminates between the indole precursor and tyrindoxyl sulphate. This is particularly advantageous for a histochemical method aimed at determining the biosynthetic origin of Tyrian purple prochromogens. Furthermore, the acid-hydrolysis method also appears to discriminate between high and low prochromogen concentrations, yielding pink and purple dye products, respectively. This specificity can be explained by the formation of dye pigment polymers at high concentration, which shifts the absorption maximum towards the red (Cooksey, 2001b).

3. 3. Trial 2: Tissue preparation

Formalin fixation coupled with paraffin embedding provides enhanced morphological definition and allows long-term storage of tissue sections. Acetone fixation of cryostat sections may also be advantageous over fresh frozen sections for similar reasons. However the effect of fixatives, solvents and processing on Tyrian purple prochromogen biochemistry and localization is currently unknown. Consequently, the impact of tissue preparation on Tyrian purple genesis from tyrindoxyl sulphate the presence of endogenous arylsulphatase and sunlight was investigated. Triplicate transverse serial sections of the hypobranchial gland from each specimen were prepared as outlined below.

Formalin fixed-paraffin embedded

Tissue was fixed in 10% neutral buffered formalin (6h), dehydrated through an ethanol series, cleared in chloroform, embedded in paraffin and sectioned at 5µm.

Prior to sunlight exposure, sections were de-waxed in xylene, and re-hydrated according the to following procedure.

1. Xylene	2min
2. Xylene	2min
3. Absolute ethanol	2min
4. Absolute ethanol	2min
5. 90% ethanol	2min
6. 70% ethanol	2min
7. Rinse sections in distilled water	
8. Incubate in direct sunlight	1h

Acetone fixed-cryostat

Tissue was fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W). Prior to direct sunlight exposure (1h), sections were fixed in acetone for 5min in a coplin jar.

Fresh-frozen cryostat

Tissue was fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm, affixed to charged slides (ProSciTech, G311SF-W) and incubated in direct sunlight for 1h.

3. 3. 1 Fixed paraffin, cryostat and fresh-frozen section comparison

Fixation in formalin and acetone prevented Tyrian purple genesis under natural conditions, although one formalin-fixed section developed a peculiar yellow-green colour, which failed to proceed to purple or pink. This is most likely due to alterations in prochromogen biochemistry or in the case of acetone fixation, prochromogen extraction. In contrast, a pink-purple product developed within the hypobranchial epithelium of fresh-frozen sections during sunlight exposure (Fig. 4). This pigmentation is presumably due to the synthesis of indole dyes after enzymatic prochromogen hydrolysis, but only in regions where arylsulphatase and tyrindoxyl sulphate co-exist. Thus, these results suggest that the proposed histochemical method should be developed with fresh-frozen tissue sections.

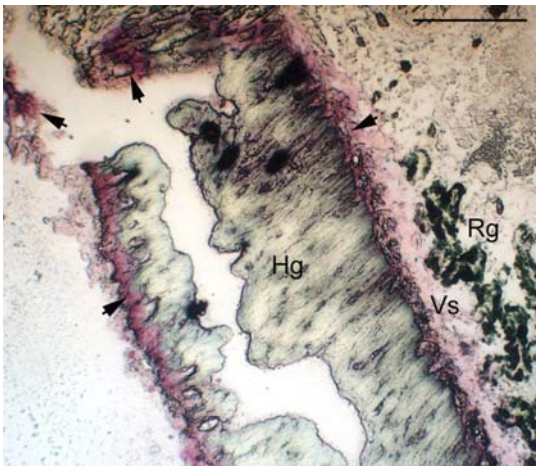

Figure 4. Tyrian purple pigment genesis (arrows) from tyrindoxyl sulphate in the presence of endogenous arylsulphatase in a fresh-frozen hypobranchial gland (Hg) section from *D. orbita*. Rg, rectal gland; Vs, vascular sinus. Scale bar = 100µm.

3. 4 Trial 3: HCl incubation period and mounting medium

In trial 1, TLC plates were exposed to concentrated (12M) HCl vapours for 5min and developed in direct sunlight for 1h. Although the acid-hydrolysis technique produced comparatively high dye yields in hypobranchial gland extracts, the effect of this visualization technique on endogenous tyrindoxyl sulphate requires investigation. Furthermore, a suitable HCl incubation period and an appropriate mounting medium need to be considered. Mounting sections prevents tissue dehydration and degradation during viewing and storage. A permanent mounting medium such as DePex mounting medium (Merck, 36125) is ideal. However, dehydration through an ethanol series and xylene incubation may be detrimental to the staining intensity or the integrity of fresh-frozen tissue. Alternatively, un-fixed sections may be mounted in glycerol for clarity of viewing and to temporarily maintain tissue hydration.

To investigate these aspects of the proposed staining protocol, triplicate transverse serial sections from the hypobranchial gland of each specimen were exposed to 12M HCl vapours within a seal chamber under fluorescent light for 1, 5, 10, 15 and 30min. Sections from each incubation trial were then mounted in either DePex mounting medium or glycerol. Additional serial sections were obtained from each specimen for comparative morphological staining in Modified Harris Haematoxylin and Eosin Y with Phloxine B (H&E) (Thompson 1966), positive and negative controls. Staining intensity and prochromogen localization was compared between each preparation and with control sections.

3. 4. 1 Tissue preparation

Tissue was fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W).

3. 4. 2 HCl incubation and mounting procedure

DEPEX mounting

1. Rinse sections in distilled water
2. Incubate in HCl vapours — 1, 5, 10, 15 or 30min
3. Rinse sections in distilled water
4. Dehydrate in 70% ethanol — 10s
5. Dehydrate in 90% ethanol — 10s
6. Dehydrate in absolute ethanol — 10s
7. Xylene — 2min
8. Xylene — Final
9. Mount sections in DePex mounting medium (Merck, 36125)

Step 2 was replaced by sunlight incubation and fluorescent light incubation for positive and negative HCl incubation controls, respectively.

Step 9 was eliminated for negative mounting controls (i.e. viewed un-mounted).

Glycerol mounting

1. Rinse sections in distilled water
2. Incubate in HCl vapours — 1, 5, 10, 15 or 30min
3. Rinse sections in distilled water

4. Mount in glycerol

Step 2 was replaced by sunlight incubation and fluorescent light incubation for positive and negative HCl incubation controls, respectively.

Step 4 was eliminated for negative mounting controls.

3. 4. 3 HCl incubation and mounting medium comparison

Hypobranchial glands within all treatment sections developed brown-pink pigmentation after HCl exposure (Fig. 5a-e). Staining was weak within sections incubated for 1min (Fig. 5a-b) and intense in 5-30min (Fig. 5c-e). In the 15min (Fig. 5d) and 30min (Fig. 5e) sections, extended HCl exposure caused tissue damage, which obscured cellular morphology.

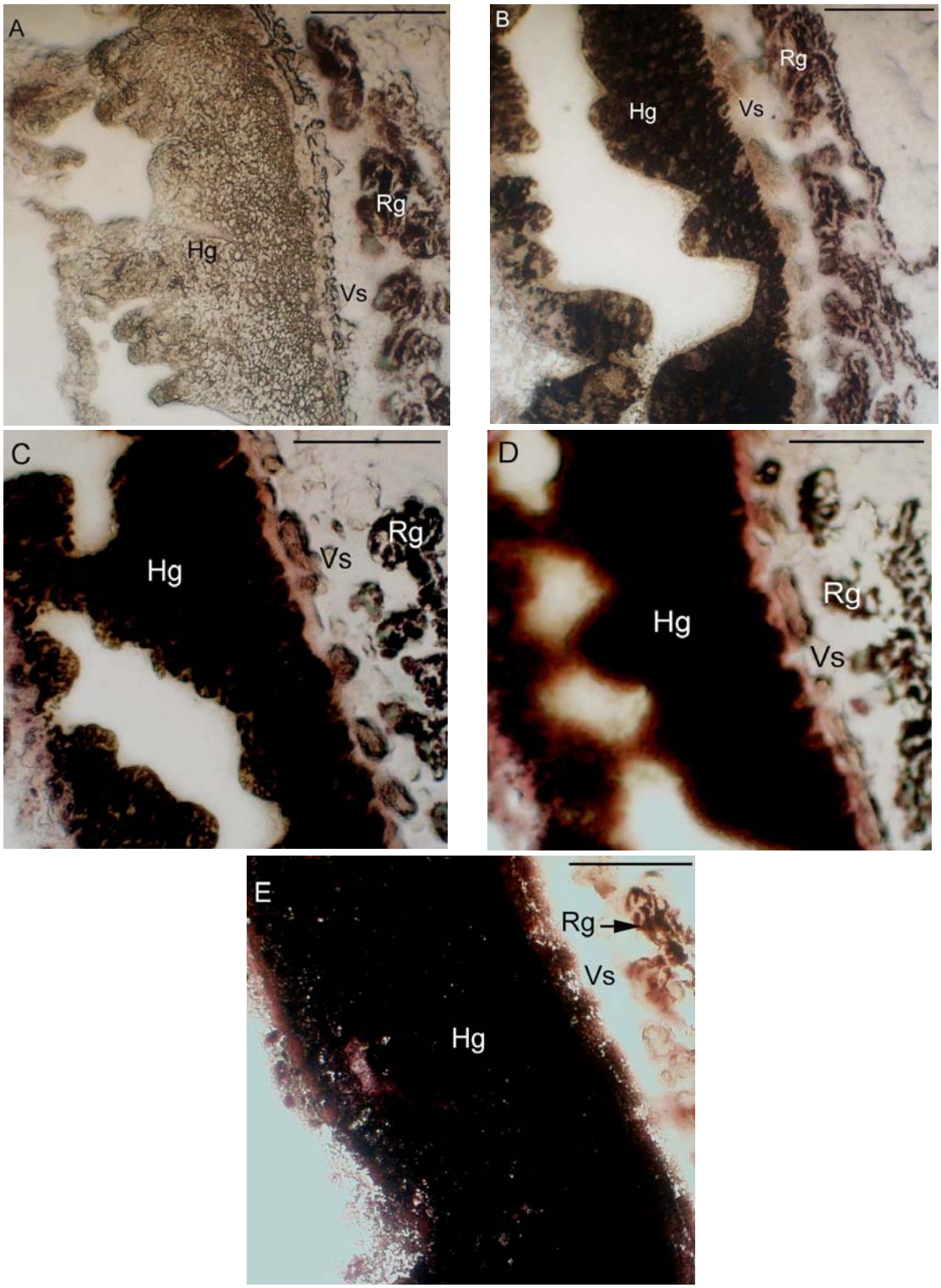

Figure 5. Brown-pink pigmentation in fresh-frozen hypobranchial gland (Hg) sections from *D. orbita* after (A) 1min, (B) 5min, (C) 10min, (D) 15min and (E) 30min HCl exposure. Rg, rectal gland; Vs, vascular sinus. Scale bars = 100µm.

Dehydration through ethanol to xylene and mounting in DePex mounting medium removed the brown pigmentation observed after HCl incubation, revealing vivid pink dye deposits (Fig. 6). The dye product of sections mounted in glycerol also gained pink pigmentation. Identical staining in regions where tyrindoxyl sulphate and arylsulphatase co-exist in positive controls suggests that pigmentation produced in treatment sections is due to acid-hydrolysis of tyrindoxyl sulphate and genesis of Tyrian purple. Overall, 10min HCl incubation of fresh-frozen tissue under fluorescent light and mounting in DePex mounting medium produced a permanent section with intense stain and good morphological integrity.

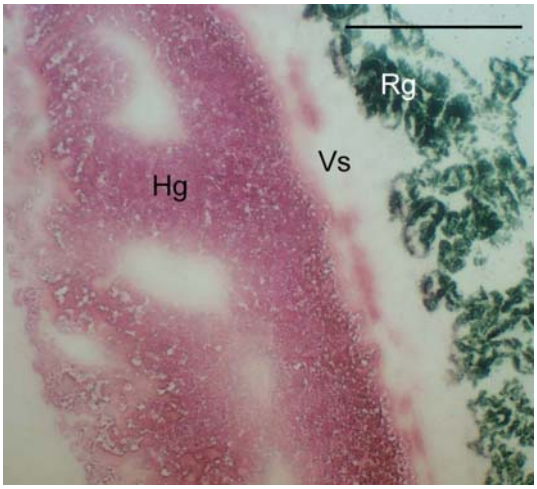

Figure 6. Vivid pink pigmentation in a fresh-frozen *D. orbita* hypobranchial gland (Hg) section after 10min HCl incubation and mounting in DePex medium. Rg, rectal gland; Vs, vascular sinus. Scale bar = 100µm.

3. 5 Trial 4: Counter staining

Depending on prochromogen concentration, acid-hydrolysis produces a pink-purple dye product. Consequently, a blue counter stain should provide sufficient contrasting morphological definition. Thus, the effect of Harris Haematoxylin

(Thompson 1966) on prochromogen staining intensity and localization was trialed. The morphological definition provided by haematoxylin staining was determined by comparison with serial sections stained in H&E (Thompson, 1966). Additional serial sections were obtained from each specimen for negative controls.

3. 5. 1 Tissue preparation

Tissue was fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W).

3. 5. 2 Staining procedure

1.	Rinse sections in distilled water	
2.	Incubate in HCl vapours	10min
3.	Rinse sections in distilled water	
4.	Stain in Haematoxylin	2min
5.	Wash in running tap water	2min
6.	Differentiate in acid-alcohol	Dip
7.	Wash in running tap water	1min
8.	Blue in lithium carbonate	2min
9.	Wash in running tap water	1min
10.	Dehydrate in 70% ethanol	10s
11.	Dehydrate in 90% ethanol	10s
12.	Dehydrate in absolute ethanol	10s
13.	Xylene	2min

14. Xylene Final

15. Mount sections in DePex mounting medium (Merck, 36125)

Steps 4-9 were eliminated for negative counter staining control sections.

3. 5. 3 Haematoxylin counter staining

Counter staining with haematoxylin prior to dehydration provided excellent morphological definition (Fig. 7), without compromising prochromogen staining intensity or localization by the “acid-hydrolysis” method.

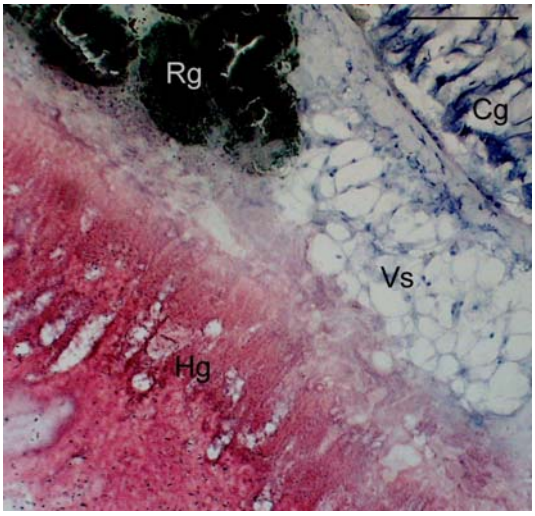

Figure 7. A fresh-frozen *D. orbita* hypobranchial gland (Hg) section stained with the acid-hydrolysis method for tyrindoxyl sulphate (pink) and counter-stained in haematoxylin. Cg, capsule gland; Rg, rectal gland; Vs, vascular sinus. Scale bar = 100µm.

3. 6 The acid-hydrolysis staining protocol for tyrindoxyl sulphate

Tissue preparation

Tissue should be fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W).

Staining procedure

1.	Rinse sections in distilled water	
2.	Incubate in HCl vapours	10min
3.	Rinse sections in distilled water	
4.	Stain in Haematoxylin	2min
5.	Wash in running tap water	2min
6.	Differentiate in acid-alcohol	Dip
7.	Wash in running tap water	1min
8.	Blue in lithium carbonate	2min
9.	Wash in running tap water	1min
10.	Dehydrate in 70% ethanol	10s
11.	Dehydrate in 90% ethanol	10s
12.	Dehydrate in absolute ethanol	10s
13.	Xylene	2min
14.	Xylene	Final
15.	Mount sections in DePex mounting medium (Merck, 36125)	

This staining protocol was reproducible and allowed localization of the prochromogen, tyrindoxyl sulphate, in *D. orbita* hypobranchial gland sections.