FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 43, No. 3, 2005
pp. 151-156

Glycoconjugate histochemistry and nNOS immunolocalization in the mantle and foot epithelia of *Tapes philippinarum* (bivalve mollusc)

Concetta Calabro[1], Maria Pia Albanese[1], Silvestro Martella[1], Patrizia Licata[2], Eugenia Rita Lauriano[1], Clara Bertuccio[1] and Aurelio Licata[1]

[1]Department of Animal Biology and Marine Ecology, Faculty of Science, and
[2]Department of Veterinary Public Health, Section of Veterinary Pharmacology and Toxicology, University of Messina, Italy

Abstract: Glycosaminoglycans and NO synthase probably regulate mucous cell secretion in the skin of *Tapes philippinarum*. We have demonstrated the presence of "protein" cells, "glycogen" cells, "phenol" cells and five types of mucous cells, with different chemical composition of the mucus in the mantle epithelium of *T. philippinarum*. The foot epithelium contained "protein" cells and two types of mucous cells. Using biotinylated lectins, in the mantle and foot epithelia we have shown specific sites for the following oligosaccharides: α-D-glucose, α-D-mannose, α-L-fucose, α-D-1,3-N-acetyl-galactosamine and α-N-acetyl-glucosamine. nNOS immunoreactivity in the intraepithelial and intradermal cells and in the mucocytes suggested a regulatory role of NO in mucus secretion, as demonstrated also in other invertebrates.

Key words: Glycoconjugates - nNOS - Mantle - Foot - *Tapes philippinarum*

Introduction

Studies on the skin of the mantle and foot of species belonging to various classes of Mollusca [18] have shown that these organisms secrete a mucus involved in numerous functions, such as locomotion, defence, offence and prey capture. The presence of mucoproteins, glycosaminoglycans and glycoproteins has been demonstrated in the secretion of specialized cells, *i.e.* "protein cells", "phenol cells", "glycogen cells" and mucocytes. In the mucus of both terrestrial and marine gastropod molluscs, sialic acid is sometimes replaced by muramic acid [4, 6, 7, 21, 24].

Nitric oxide (NO) is present in the central and peripheral nervous systems, and is also involved in modulation of exocrine secretion in the skin of some mammals [22, 23] and in muscle cells of some primitive molluscs [19]. NO was also recently demonstrated in the tegument of invertebrates [15, 16] and in the surface epithelia of the skin and gills of lower vertebrates [29, 30, 31], where it plays a modulatory role in mucus secretion.

Previous studies of several species of Gastropoda Prosobranchia have shown that the mucous cells in the skin of the mantle and foot vary in number and localization within the same species [2, 3, 7].

Recently we carried out a phylogenetic study of the Indopacific bivalve mollusc *Tapes philippinarum*. This species was introduced to the upper Adriatic Sea for aquacultural purposes in 1983, replacing the native *Tapes decussatus* [27], and was later introduced to the brackish Lake Faro (Messina).

The aim of the present immunohistochemical study was to investigate the role of NO in the functional activities of the tegument of the foot and mantle of *T. philippinarum*. Moreover, using histochemical methods and exogenous lectins, we made an attempt to characterize the chemical nature of the mucus, also in comparison with that of the related species *Tapes decussatus* [9].

Materials and methods

Individuals of *Tapes philippinarum* were collected from Lake Faro (Messina). They were fixed in Bouin, Carnoy and 4% paraformaldehyde and embedded in paraplast. Sections were stained by the Mallory method modified by Ignesti (Carazzi hemalum , 1% acidic

Correspondence: A. Licata, Dept. Animal Biology and Marine Ecology, University of Messina, Contrada Sperone 31, 98166 S. Agata Messina, Italy; e-mail: alicata@unime.it

fuchsin, Mallory's solution) and Galgano I (Mayer's hemalum, 0,1% acidic fuchsin, 1% phosphomolybdic acid, Mallory's solution).

The following histochemical methods (Table 1) demonstrating proteins, glycoproteins and acidic proteoglycans were used.

Proteins: bromophenol blue method [20], ninhydrin-Schiff, chloramine T-Schiff, performic acid-Schiff, Morel and Sisley, Millon, and Adams reactions, diazoreaction [8], Sakaguchi reaction [26].

Glycoproteins: alcian blue-PAS (AB-PAS) reaction according to McManus [17]; dimedone-PAS reaction.

Acidic proteoglycans: AB (pH 1); AB (pH 2.5).

Distinction between glycoproteins and acidic proteoglycans: AB-PAS (pH 1 and 2.5).

Distinction between acid proteoglycans with different acidities: AB-C.E.C. (critical electrolyte concentration): 0,05% AB, inhibition of alcianophilia at graded molarities of 0.5-1 M $MgCl_2$ in acetic buffer (pH 5.8) weak methylation-AB (pH 2.5); strong methylation-AB (pH 2.5); weak methylation-saponification-AB (pH 2.5); strong methylation-saponification-AB (pH 2.5). Acid hydrolysis in 0.1 M acetate buffer (pH 2.5) for 4 h at 60˚C followed by AB (pH 2.5) also without saponification.

Methods of enzymatic digestion: PAS-amylase; AB-neuraminidase (pH 2.5, from *Clostridium perfrigens*) with and without saponification; testicular hyaluronidase.

Specific sugar residues: biotinylated lectins and avidin-biotin-peroxidase method (ABC) [13] was employed. The biotinylated lectins used (Vector Laboratories, Burningame, California, USA), their origin, specificity and inhibiting sugars are reported in Table 2.

Controls were performed by incubating the sections with the specific sugar.

To reveal nNOS, tissue sections were incubated overnight at 4˚C in a humid chamber with polyclonal rabbit anti-human brain nNOS antibodies, diluted 1:200 (nNOS type I antibodies; code 606-259-1550; Biomol, Hamburg, Germany). The sections were then washed in PBS and incubated for 2 h with a goat anti-rabbit IgG-peroxidase conjugate (1:100; Sigma, Munich, Germany). Peroxidase activity was visualized by incubation of the sections for 5-12 min at room temperature in a solution of 0.015% 3,3'-diaminobenzidine in 0.01 M Tris buffer (pH 7.6) containing 0.005% H_2O_2. Negative controls included omission of the primary antibody or its substitution with non-immune rabbit serum.

Table 1. Conventional histochemical methods for visualization and identification of glycoconjugates (GCs)

Procedure	GCs revealed
PAS	Glycoproteins
AB pH 1	Proteoglycans with sulphate groups
AB pH 2.5	Proteoglycans with carboxyl groups
AB pH 2.5/PAS	Proteoglycans with carboxyl groups and glycoproteins
Neuraminidase digestion	Sialic acid
AB (C.E.C.)	Sialic acid

Table 2. Lectins employed and their major binding specificities

Lectin	Origin	Carbohydrate specificity	Inhibitor sugar
ConA	*Canavalia ensiformis*	α-D-mannose, α-D-glucose	D-Mann
PHA-L	*Phaseolus vulgaris*	oligosaccharides	D-GalNAc
PNA	*Arachis hypogea*	D-galactose	D-gal
SBA	*Glycine max*	N-acetyl-D-galactosamine> D-galactose	D-galNAC
WPA	*Lotus tetragolobus*	α-L-fucose	L-fucose
WGA	*Triticum vulgaris*	N-acetyl-D-galactosamine> sialic acid	D-GlcNac

Results

In *Tapes philippinarum*, the skin of the mantle is morphologically and histologically different from that of the foot. The simple epithelium of the foot, raised into more numerous folds on the dorsal surface, is composed of ciliated columnar cells on the dorsal face and of nonciliated cuboidal cells on the ventral face. The connective tissue consists of intertwined collagen fibres mixed with bundles of muscle fibres. Mucocytes are evident only on the dorsal face (Fig. 1).

In the mantle, the skin consists of rather loose connective tissue, crossed by muscle fibres. On the edge of the mantle, there are four deep folds where the external simple epithelium is composed only of nonciliated cuboidal cells, while in the thickness of the folds there are also goblet cells. The mucous cells contain glycoproteins and proteoglycans with acidic groups (Fig. 2)

The histochemical methods revealed "protein" cells among epithelial cells in both the mantle and foot. The diazoreaction carried out in alkaline medium with Fast blue showed "phenol" cells only in the mantle. PAS-positive cells showing the presence of glycoproteins were only found in the mantle. The staining was not present in sections treated with α-amylase, indicating the presence of "glycogen" epithelial cells coexisting with mucous cells in the underlying connective tissue. Moreover, the reactions to show acid glycosaminoglycans revealed five types of mucous cells in the mantle: cells with glycoproteins only, with sulphated acidic proteoglycans, with proteoglycans containing carboxyl groups, and with glycoproteins and proteoglycans containing carboxyl groups (Fig. 3).

Some mucous cells of the foot epithelium contained carboxyl acidic proteoglycans (Fig. 4) others also glycoproteins (Fig. 5).

Sialic acid and hyaluronic acid were not found in the tegument of the mantle and foot.

Use of the ConA, PHA-L, PNA, WPA and SBA lectins showed that the secretion of the intraepithelial and intradermal mucous cells of the mantle and foot were positive for α-D-glucose and α-D-mannose (Fig. 6), oligosaccharides (Fig. 7), α-D-gal and (1-3)-D-Gal-Nac (Fig. 8), α-L-fucose (Fig. 9) and N-acetyl-D-galactosamine or D-galactose (Fig. 10). In the surface epithelium of the mantle, few neuroendocrine cells were

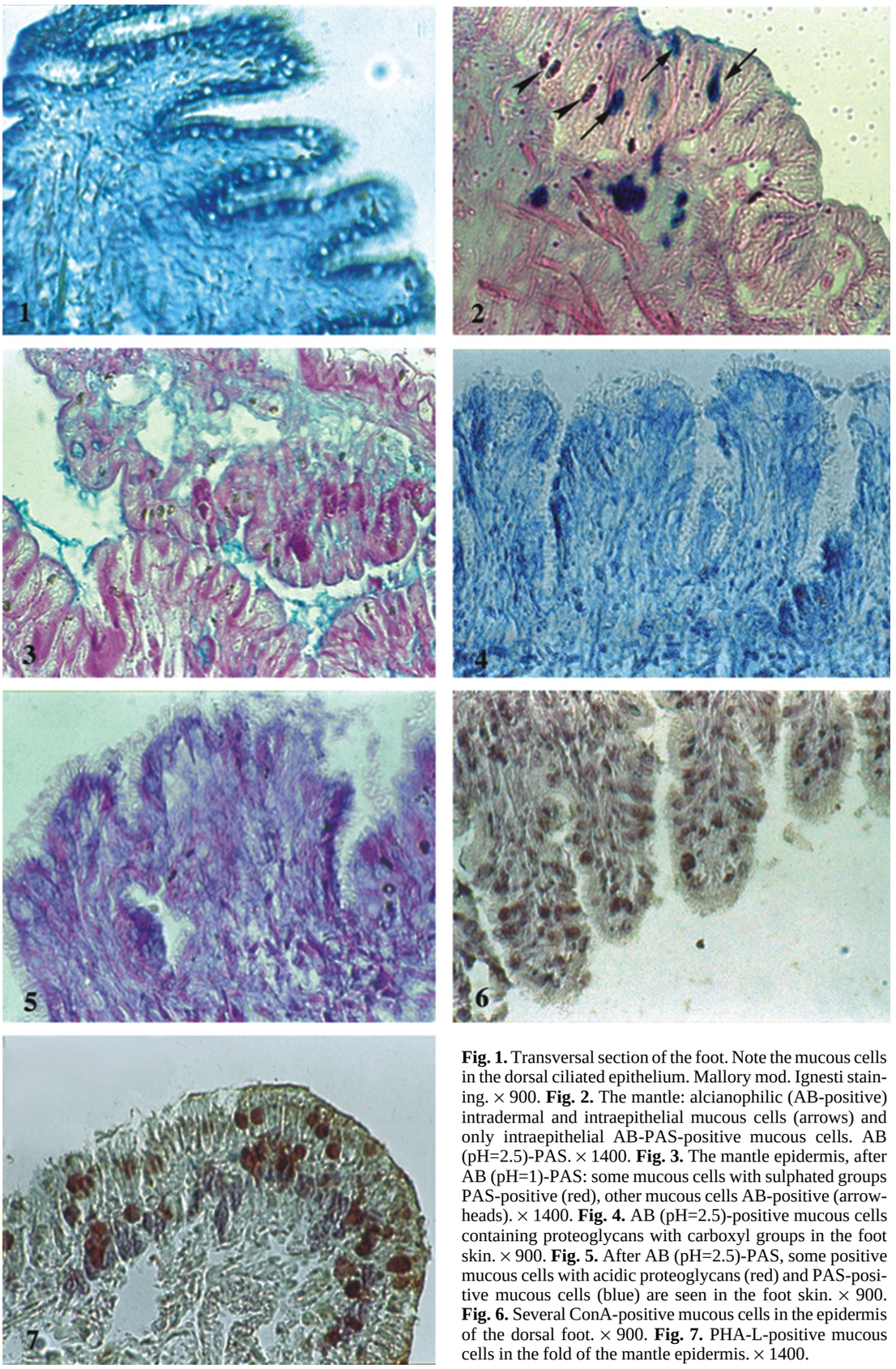

Fig. 1. Transversal section of the foot. Note the mucous cells in the dorsal ciliated epithelium. Mallory mod. Ignesti staining. × 900. **Fig. 2.** The mantle: alcianophilic (AB-positive) intradermal and intraepithelial mucous cells (arrows) and only intraepithelial AB-PAS-positive mucous cells. AB (pH=2.5)-PAS. × 1400. **Fig. 3.** The mantle epidermis, after AB (pH=1)-PAS: some mucous cells with sulphated groups PAS-positive (red), other mucous cells AB-positive (arrowheads). × 1400. **Fig. 4.** AB (pH=2.5)-positive mucous cells containing proteoglycans with carboxyl groups in the foot skin. × 900. **Fig. 5.** After AB (pH=2.5)-PAS, some positive mucous cells with acidic proteoglycans (red) and PAS-positive mucous cells (blue) are seen in the foot skin. × 900. **Fig. 6.** Several ConA-positive mucous cells in the epidermis of the dorsal foot. × 900. **Fig. 7.** PHA-L-positive mucous cells in the fold of the mantle epidermis. × 1400.

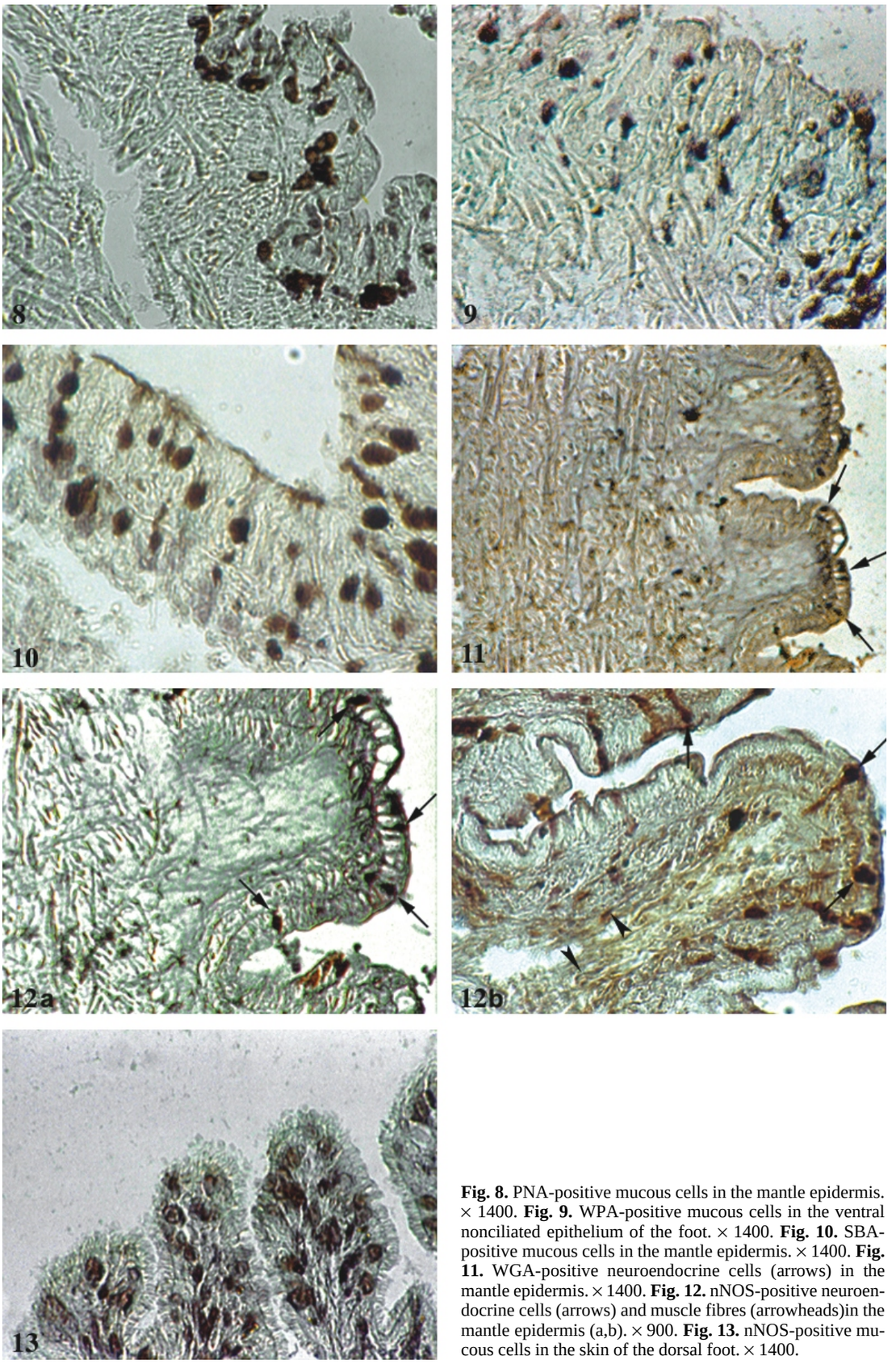

Fig. 8. PNA-positive mucous cells in the mantle epidermis. × 1400. **Fig. 9.** WPA-positive mucous cells in the ventral nonciliated epithelium of the foot. × 1400. **Fig. 10.** SBA-positive mucous cells in the mantle epidermis. × 1400. **Fig. 11.** WGA-positive neuroendocrine cells (arrows) in the mantle epidermis. × 1400. **Fig. 12.** nNOS-positive neuroendocrine cells (arrows) and muscle fibres (arrowheads)in the mantle epidermis (a,b). × 900. **Fig. 13.** nNOS-positive mucous cells in the skin of the dorsal foot. × 1400.

positive for WGA (Fig. 11). In the mantle epithelium, nNOS-positive nonmucous cells were found (Figs. 12a-b), while in the foot epithelium only the mucous cells were nNOS-positive (Fig. 13).

Discussion

The skin of the mantle and foot of *Tapes philippinarum*, structured differently in the two regions, presents similarities with that of *Mercenaria mercenaria* [12], *Venus verrucosa* [7] and *Ensis siliqua* [1]. The cytochemical investigation of the mantle and foot epithelia revealed the presence of "protein" cells, "glycogen" cells and a few "phenol" cells only in the mantle, and five types of mucous cells. Previous histochemical studies of both the skin and mantle of several species of Lamellibranchia demonstrated the presence of tyrosine, tryptophan and amine radicals in the "protein" cells [1, 7, 9, 25]. However, these substances are not present in *T. philippinarum*. The presence of "phenol" cells only in the mantle epithelium partially agrees with the situation in other species. The presence of "glycogen" cells, in addition to mucocytes, only in the mantle epithelium is interesting; in other species, these cells are found only in certain areas of the epithelium [1, 6, 9, 25]. The mucous cells of the mantle epithelium contain glycoproteins as well as acidic proteoglycans with both sulphur groups and carboxyl groups either alone or coexisting with glycoproteins. In the tegument of the foot, the secretory material of these cells was positive for either sulphated acidic proteoglycans or for acidic proteoglycans with carboxyl groups. The chemical composition of the mucus in the mantle and foot agrees with what was observed in *Tapes decussatus* [9] and in *Ensis siliqua* [1].

Conventional histochemical techniques failed to reveal sialic acid in the mucous cells. However, specific sites for sialic acid in these cells were shown with WGA. Sialic acid was found to be absent in previously studied molluscs, with the exception of the freshwater species *Parreysia corrugata* [14]. This absence from mucous cells [5] has been explained by the lack of enzymatic systems responsible for the incorporation of sialic acid residues into the mucus. The presence of N-acetyl-glucosamine sites in mucocytes of the skin of *Tapes philippinarum*, revealed with WGA, suggests that sialic acid is masked by other organic compounds that prevent its detection by conventional histochemical techniques. The presence of glycoconjugates positive to ConA, PHA-L, SBA and PNA in the mucus of both the foot and mantle could be related to the role these molecules play in the recognition and elimination of pathogens. They could be part of a defence system contributing to a protective mechanism in the mucous epithelia of invertebrates. PNA positivity in the mucus also indicates an involvement in electrolyte homeostasis. Glycoconjugates in the mucus positive to PNA and SBA also regulate the functional integrity of membrane receptors in the transduction of membrane signals [28].

The presence of nNOS in intraepithelial and intradermal mucous cells of the foot of *T. philippinarum* agrees with what was observed in skin glands of domestic mammals [10, 22, 23] and in the earthworm epidermis [15], where NO is involved in the modulation of mucus secretion with paracrine activity and in cell-cell communication. In the mantle tegument, the mucous cells were negative to nNOS, while the nNOS-positive intraepidermal cells are probably neuroendocrine in nature, what is in line with previous studies of the epidermis of *L. terrestris* [15]. The presence of neuroactive substances such as NO in specialized cells of the mantle epidermis can be explained by the functional adaptation of the tegument of invertebrates during evolution, especially in relation to its secretory activity. The presence of sialic acid, revealed by WGA, and the nNOS positivity in neuroendocrine cells confirm previous findings in the earthworm [15].

WGA-binding sites in the neuroendocrine cells may be related to the release of secretory substances which occurs by exocytosis in the paraneuronal cells [11]. In the foot epithelia, nNOS-positive mucous cells probably release the mucus by autocrine activity. In the mantle epithelia, nNOS-positive neuroendocrine cells secrete NO inducing, by paracrine function, the mucous cells to release the mucus.

References

[1] Albanese MP, Calabro C, Lo Cascio P, Zaccone G (1977) Ricerche strutturali ed istochimiche sulle cellule mucose del tegumento di *Ensis siliqua* L (Moll Lamell). Riv Biol Norm Patol 3: 241-258

[2] Albanese MP, Calabro C (1978) Studio strutturale ed istochimico sul mantello e sul piede di *Pisania maculosa* LMK. (Moll Gast Pros). Riv Biol Norm Patol 4: 219-245

[3] Albanese MP, Calabro C, (1982) Ricerche strutturali ed istochimiche sul mantello e sul piede di *Lunatia guglielmini* Paraudeau (Moll Gast Pros). Riv Biol Norm Patol 8: 3-14

[4] Albanese MP, Calabro C (1996) Studio strutturale ed istochimico sul mantello e sul piede di *Natica millepunctata* LMK (Moll Gast Pros). Riv Idrobiol 35: 87-99

[5] Bolognani-Fantin AM (1968) Sull'assenza degli acidi sialici nelle mucine cutanee dei Molluschi. Boll Zool 35: 374 -375

[6] Bolognani-Fantin AM, Vigo E, Gervaso MV (1969) La mucinogenesi nei Molluschi.VI.Caratteristiche dei tipi cellulari presenti nel piede e nel mantello di alcune specie di Lamellibranchi. Riv Istochem Norm Patol 15: 345-370

[7] Bolognani-Fantin AM, Ottaviani E (1990) N-acetylmuramic acid in Mollusca Gastropoda: a histochemical and immunocytochemical study. Histochem J 22: 475-479

[8] Bonucci E (1981) Manuale di Istochimica, Lombardo Editore, Roma

[9] Capurro S, Grossi G (1961) Ricerche di qualificazione istochimica della cute di *Tapes decussatus* L nei riguardi del materiale mucopolisaccaridico. Riv Ital Istochem 7: 53-76

[10] Dippel EJ, Mayer B, Scanfelder G, Czarnetski BM, Paus R (1994) Distribution of costitutive nitric oxide synthase immu-

noreactivity and NADPH-diaphorase activity in murine telogen and anagen skin. J Invest Dermatol 103: 112-115
[11] Fujita T, Kanno T, Kobayashi S (1988) The paraneuron. Springer, Tokyo
[12] Hillmann RE (1964) Histochemistry of mucosubstances in the mantle of the clan *Mercenaria mercenaria*. A glycosaminoglycan in the first marginal fold. Trans Am Microsc Soc 87: 361-367
[13] Hsu SM, Raine L (1982) Versatility of biotin-labeled lectins and avidin-biotin-peroxidase complex for localization of carbohydrate in tissue sections. J Histochem Cytochem 30: 157-161
[14] Kale GS, Patil VY (1976) Histochemistry of mucosubstances in the mantle of fresh water mussel *Parrejsia corrugata var. nagpoorensis* (Lea). I Mucosubstances of the inner marginal fold. Folia Histochem Cytochem 14: 143-148
[15] Licata A, Ainis L, Martella S, Ricca MB, Licata P, Lauriano ER, Zaccone G (2002) Immunohistochemical localization of nNOS in the skin and nerve fibres of the earthworm *Lumbricus terrestris* L (Anellida Oligochaeta). Acta Histochem 104: 289-295
[16] Licata A, Ainis L, Martella S, Ricca MB, Licata P, Pergolizzi S, Calabro C, Zaccone G (2003) Immunohistochemical localization of nNOS and VIP in the mantle integument of the mussel, *Mytilus galloprovincialis.* Acta Histochem 105: 143-149
[17] MacManus JFA (1948) Histological demonstration of mucin after periodic acid. Nature 15: 202
[18] Manfredi-Romanini MG, Bolognani-Fantin AM (1992) Polysaccharides and glycoconjugates in invertebrates and in non mammalian vertebrates. In: Handbuch der Histochemie, Graumann W [Ed], Vol 2/V, Springer, Berlin
[19] Martinez A (1995) Nitric oxide synthase in invertebrates. Histochem J 27: 770-776
[20] Mazia D, Brewer PA, Alfert M (1953) The cytochemical staining and measurement with mercuric bromophenol blue. Biol Bull 104: 57-67
[21] Menghi G, Ottaviani E, Accili D, Bolognani-Fantin AM (1991) Identification of muramyl derivates in the Mollusca Gastropoda tissue. Histochemistry 96: 209-213
[22] Meyer W, Neurand K, Baetles T, Kojda G, Mayer B (1994) Demonstration of NADPH-diaphorase (NO-synthase) in the apocrine and eccrine skin glands of domesticated mammals. Cell Mol Biol 40: 175-181
[23] Meyer W, Neurand K, Godynicki S, Koida G, Mayer B (1996) Demonstration of NADPH-diaphorase (NO synthase) in sebaceus gland of the glandular capillary net. Cell Mol Biol 42: 241-248
[24] Ottaviani E, Bolognani-Fantin AM, Bolognani L (1990) Muramic acid as a glycoconjugate component in Mollusca Gastropoda. Comp Biochem Physiol 96 B: 627-632
[25] Palladini G, Lauro G (1966) Ricerche istotopochimiche sulle secrezioni mucose del piede di *Mytilus galloprovincialis* Lam. Mon Zool Ital 74: 49-66
[26] Pearse AGE (1968) Histochemistry, theoretical and applied, Vol 1**,** J. Churchill, London
[27] Pellizzato M (1990) In: *Tapes philippinarum*, Biologia e sperimentazione. Ed ESAV, pp 147-170
[28] Shinohara H (1977) On glycoproteins. Biochemistry 49: 1219-1237
[29] Zaccone G, Fasulo S, Ainis L, Licata A (1997) Paraneurons in the gills and airways of fishes. Microsc Res Tech 37: 4-12
[30] Zaccone G, Kapoor BG, Fasulo S, Ainis L (2001) Structural, histochemical and functional aspects of the epidermis of fishes. Adv Mar Biol 40: 255-347
[31] Zaccone G, Mauceri A, Ainis L, Licata A, Fasulo S (2003) Nitric oxide synthase in the gill and air sac of of the Indian catfish, *Heteropneustes fossilis.* In: Catfishes. Kapoor BG, Arratia G, Chardon M, Diogo R [Eds], Oxford and IBH Publishing, New Delhi-Calcutta, pp 771-788

Received: December 12, 2004
Accepted after revision: February 28, 2005