

Peptidergic and Adrenergic Innervation of the Lachrymal Gland in the Euryhaline Turtle, *Malaclemys terrapin*

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ABSTRACT The controlling factors of lachrymal gland secretions were examined in the euryhaline turtle, *Malaclemys terrapin*. Histochemical and immunocytochemical methods were used to localize some of the possible neurotransmitters involved.

There was no immunoreactivity to choline acetyltransferase, the enzyme synthesizing acetylcholine, nor did the histochemical technique for acetylcholinesterase produce positive results. Immunofluorescence and immunoperoxidase labels revealed vasoactive intestinal peptide (VIP)- and neuropeptide Y (NPY)-like immunoreactivity in high concentrations surrounding the secretory tubules and ducts. Substance P produced a weak immunoreactivity in the interstitial space surrounding the ducts. Dopamine β -hydroxylase, the enzyme synthesizing norepinephrine and epinephrine, was localized around the blood vessels. Immunogold labeling confirmed the presence of VIP- and NPY-like reactivity in nerve varicosities close to the basement membrane of the secretory epithelium, and double-labeling showed VIP and NPY are co-localized within the same nerve terminals.

The results suggest that the secretory epithelium may be primarily under peptidergic control while the vascular system is under adrenergic control. This is possibly a new pattern of innervation for exocrine glands and may be related to the particular function of this salt gland in an euryhaline turtle. © 1995 Wiley-Liss, Inc.

The lachrymal gland of the euryhaline turtle, *Malaclemys terrapin*, has an ultrastructure resembling other secretory epithelia known to be involved in ion regulation. Certain morphological features, which include extensive basolateral membrane folds and abundant mitochondria, indicate this gland in *Malaclemys* may be involved in ion regulation (Cowan, '71; Peaker, '79). Physiological experiments have indicated that a secretion with a concentration of sodium chloride greater than seawater can be produced. The ability to generate "free water" may represent one of several adaptations which allows *Malaclemys* to survive in seawater while maintaining a blood osmolality of approximately 300 mosm.

Some of the other epithelial tissues involved in the excretion of excess sodium have been more thoroughly studied. In the elasmobranchs, the rectal gland can be examined using perfusion methods. A model for the mechanism of secretion in this gland has been proposed, and control by peptidergic nerves has been suggested (Shuttleworth and Thorndyke, '84; Silva et al., '87). A mechanism of secretion involving cholinergic

innervation has also been proposed for the avian salt gland (Schmidt-Nielsen and Fange, '58; Ash et al., '69), and for the lachrymal gland in *Chelonia mydas* (Schmidt-Nielsen and Fange, '58).

The small size of the lachrymal gland in *Malaclemys* and the microscopic secretory ducts has so far precluded the acquisition of evidence for any detailed model of secretion. However, pharmacological experiments in vivo using a variety of cholinergic agents have given no evidence of cholinergic innervation. Unpublished observations of ours provide evidence of adrenergic innervation, and one experiment indicated a possible role for peptide neurotransmitters.

In the present study, indirect immunofluorescent, immunoperoxidase, and immunogold-labeling techniques were used to substantiate the in vivo physiological experiments by attempting to localize neurons containing acetylcholine, cat-

Received May 8, 1995; revision accepted August 29, 1995.

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echolamines, or various peptide neurotransmitters in the lachrymal gland of *Malaclemys*. As further support for the presence or absence of acetylcholine, the histochemical technique for acetylcholinesterase was carried out.

The results do support the physiological findings and suggest that the secretory epithelium may be primarily under peptidergic control while the vascular system is under adrenergic control. There was no localization of cholinergic neurons. This uncommon pattern may relate to the possible multiple roles of the lachrymal gland in *Malaclemys*.

MATERIAL AND METHODS

Animals

The turtles, *Malaclemys terrapin*, were obtained from a source near Chincoteague, Virginia, and were kept in fiberglass tanks in either fresh water or seawater (Instant Ocean). The experimental animals were either male or female and weighed from 200 to 800 g. Since it was not known if salinity changes may affect neurotransmitter profiles in the lachrymal gland, initial experiments included turtles from fresh water and those acclimated for 2 weeks to seawater.

Animals were decapitated, and the lachrymal gland was dissected from the orbit. The tissue was dissected under the appropriate fixatives and treated as outlined in the following sections. In some cases, rat brain and/or turtle brain were evaluated along with the experimental tissue as positive controls for the antibodies and the technique.

Antisera and reagents

The primary antibodies, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), substance P, serotonin, and dopamine β -hydroxylase, were all polyclonal and were obtained from Incstar, Stillwater, MN. A monoclonal antibody to choline acetyltransferase (ChAT) was purchased from Incstar, and a polyclonal, ChAT antibody was obtained from Chemicon, Temecula, CA.

Fluorescein-conjugated secondary antibody (goat anti-rabbit IgG-fluorescein) was purchased from Jackson Immunoresearch, West Grove, PA and the Vectastain ABC (avidin-biotin complex) kit was obtained from Vector Laboratories, Burlingame, CA. All synthetic antigens came from Sigma Chemical Company (St. Louis, MO).

The 14-nm protein A-gold complex was made in our laboratory according to the protocol of Bendayan

(89) and the 5-nm protein A-gold complex was purchased from Sigma Chemical Company.

Immunohistochemical technique

The tissue was cut by a vibrating microtome into sections, 50 to 100 μ m thick, and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. All solutions and incubations were at 4°C, and rinsing involved five washes in 1 hour. Immunoreactivity for VIP, NPY, substance P, serotonin, and dopamine β -hydroxylase was made visible by fluorescence staining. The tissue was rinsed with phosphate-buffered saline (PBS) (pH 7.4) containing 0.3% Triton X-100 and then incubated for 24 hours in rabbit primary antiserum diluted (1:200) with PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA). After rinsing in PBS, the sections were incubated in fluorescein-labeled goat anti-rabbit IgG (1:40) for 24 hours. After a final rinse the sections were mounted in a glycerol:PBS (3:1) buffer on glass slides and examined in a Leitz Orthoplan epifluorescent microscope. Each antibody was tested on four animals, two from fresh water and two from seawater. An additional trial, using the peroxidase label, was performed with VIP, NPY, dopamine β -hydroxylase, and serotonin. Immunoreactivity for choline acetyltransferase was made visible by the peroxidase label, avidin-biotin complex. The tissue was pretreated first with 0.3% H₂O₂ in PBS for 30 minutes, rinsed with PBS, and then given a second pretreatment for 4 hours in PBS containing 10% normal goat serum and 0.5% Triton-X 100. The sections were incubated for 4 days in rabbit anti-ChAT, diluted 1:500, 1:1,000, 1:2,000, with PBS containing 0.5% Triton-X 100. Following several washes, sections were exposed to biotinylated goat anti-rabbit IgG (Vectastain ABC Kit) for 2 days at room temperature, rinsed, and treated to tertiary reagent, avidin complex, for another 2 days at room temperature. After rinsing, the enzyme activity was revealed by pretreating the sections in Tris-HCl buffer containing 0.5% 3,3'-diaminobenzidine(DAB) for 20 minutes and then treatment with DAB and 0.1% H₂O₂ under visual observation for 5 to 10 minutes. Sections were dehydrated and mounted on slides with Epon-Araldite. Three animals were examined for immunoreactivity to the ChAT antibody.

To test the specificity of the method and the antisera, three controls were performed. These were 1) omission of the primary antibody, 2) substitution of the primary antibody with normal host serum (1:200), and 3) substitution of the primary antibody

with antisera preabsorbed with its corresponding antigen (100–200 $\mu\text{m}/\text{ml}$). Untreated, fixed sections were examined for autofluorescence, and fixed tissue, treated with the peroxidase chromogen, was examined for endogenous peroxidase. In addition to the above controls, one preabsorbed experiment was performed to check for possible cross-reactivity between the VIP and NPY antibodies.

All of the controls for the immunofluorescent and immunoperoxidase protocols and for acetylcholinesterase indicated specificity in the above results. Autofluorescence and endogenous peroxidase in the lachrymal gland did not interfere with their respective labeling patterns in the nerve terminals in the interstitium. Positive controls in the rat and/or turtle brain confirmed that the techniques were working. The cross-reactivity test showed little to no cross-reactivity between NPY and VIP.

Conventional transmission electron microscopy

Tissues were fixed for 2–4 hours at 4°C in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) or in Karnovsky's fixative (Karnovsky, '65); dehydrated in acetone, and embedded in Epon-Araldite. Silver to gold-colored sections were collected onto formvar-coated grids, post-stained with uranyl acetate and lead citrate, and examined at 80 kV in a Philips 400T transmission electron microscope.

Immunogold labeling

For immunogold labeling, the material was fixed in 0.1% glutaraldehyde and 1.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at 4°C, dehydrated in ethanol, and embedded Lowicryl K4M according to the protocol of Bendayan ('84). Sections were collected on formvar-coated and uncoated nickel grids. The 14-nm colloidal gold suspension was prepared according to Frens ('73) and the complex was formed in a centrifuge after Bendayan ('84). Antisera for VIP, NPY, substance P, and dopamine β -hydroxylase were used in conjunction with the protein A-gold complex. Double-labeling experiments were carried out on 300-mesh nickel grids. The first antisera was labeled on face A with the 14-nm gold complex, and then after drying, face B was incubated in the second antisera and labeled with the 5-nm gold complex.

Three controls were used in the immunogold study. They are 1) the omission of the primary antibody, 2) substitution with normal host se-

rum, and 3) incubation in unlabeled protein A followed by the complete protein A-gold complex. All three of the methodology controls used in this study demonstrated the specificity of the immunogold label.

Acetylcholinesterase histochemistry

The tissue was fixed for 24 hours in 4% paraformaldehyde, rinsed in a phosphate buffer, and pretreated with 0.1% hydrogen peroxide. The fixed tissue was incubated in acetylthiocholine iodide, after the protocol of Tago et al., ('86), rinsed, and then reacted with the chromogen, DAB, and hydrogen peroxide. For light microscopy, sections were mounted in a buffer:glycerol solution and sealed with a coverslip. Two controls were used in this technique: 1) the omission of the acetylthiocholine substrate and 2) the substitution of butyrylthiocholine iodide for acetylthiocholine plus the addition of an inhibitor, tetraisopropylpyrophosphoramidate (iso.OMPA, Sigma).

RESULTS

General gland structure

The lachrymal gland consists of a system of branched secretory tubules or acini lined by simple columnar epithelial cells surrounding a central lumen (Fig. 1A). The most prominent features of the principal secretory cells observed were abundant mitochondria, numerous plications of the lateral membrane surface, and variable intercellular spaces (Fig. 1B). Cellular extensions into the lumen are short and sparse, lateral plications are extensive and interdigitate with those of neighboring cells, while on the basal surface plications are few. The cell is usually seen lying on a basement membrane separating the secretory tubular system from the underlying interstitial layer.

Connective tissue, small arterioles, venules, capillaries, small nerve fibers, and pigment granules were found in the interstitium surrounding the secretory tubules. No nerve fibers or nerve terminals were observed inside the basement membrane of the secretory tubules. The nerve terminals contained dense-cored vesicles of approximately 100 nm diameter and smaller vesicles of 50 nm diameter with electron-lucent contents (Fig. 1C). Pigment cells containing dark granules were observed within the interstitium in discrete areas throughout the gland. The granules were from 300 nm to 1 μm in diameter, oval or round, and brown-black or electron-dense.

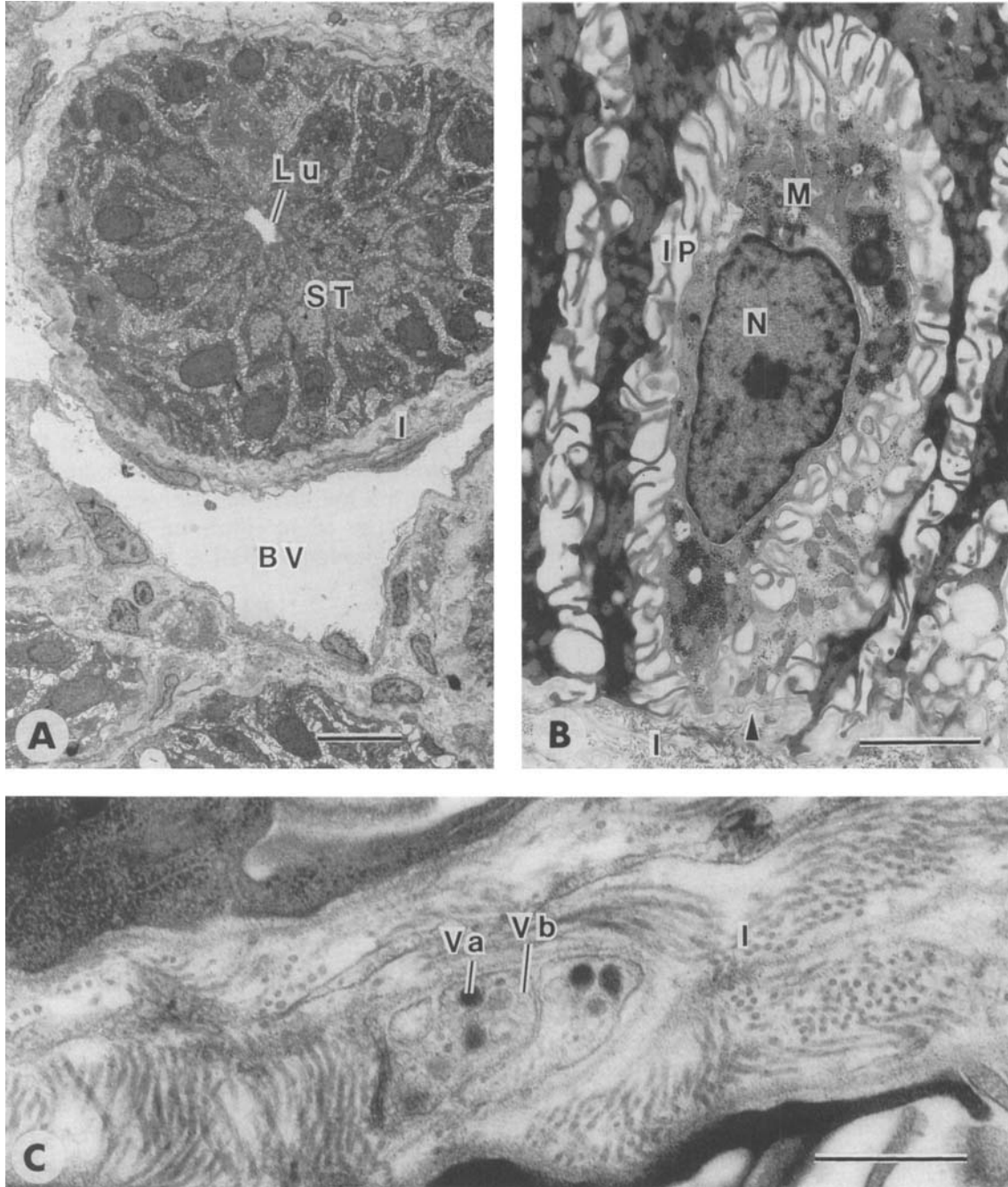


Fig. 1. Morphological features of lachrymal gland in *Malaclemys terrapin*. ST, secretory tubule; Lu, lumen; I, interstitial space; BV, lumen of blood vessel; M, mitochondria; N, nucleus; IP, intercellular processes. **A:** TEM of a secretory tubule (acinar) surrounded by interstitium and adjacent to a

blood vessel. **B:** TEM detail of a secretory (principal) cell abutting on basement membrane (arrowhead). **C:** Small nerve ending in the interstitium containing dense-cored vesicles (Va) and smaller, electron-lucent neurosecretory vesicles (Vb). A: bar = 10 μ m; B: bar = 2 μ m; C: bar = 0.5 μ m.

Immunohistochemistry

Immunofluorescent experiments with the VIP antibody revealed a strong label in the nerve fibers surrounding the secretory tubules and ducts

(Figs. 2A,B). In Figure 2A, the extent of labeling is evident in the interstitial space surrounding the secretory tubules and ducts and is confined to the fine nerve fibers and their varicosities.

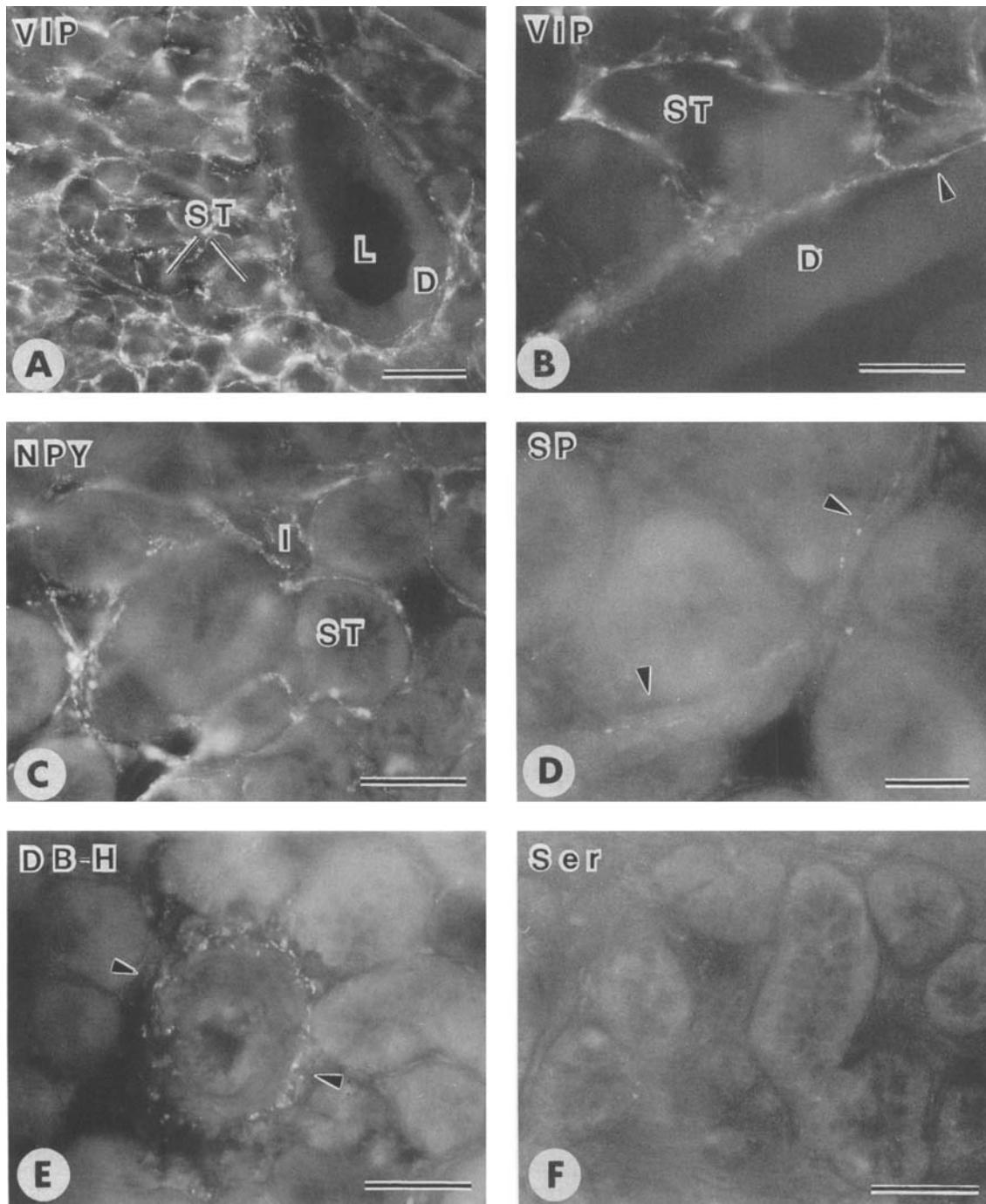


Fig. 2. Adrenergic and peptidergic immunofluorescence in the lachrymal gland. ST, secretory tubules; D, duct; L, lumen; I, interstitium. **A:** Micrograph showing VIP-like immunoreactivity in the interstitium surrounding the secretory tubules and ducts. **B:** Fine nerve fibers (arrowhead) are seen along the interstitial space at the basal end of a duct. **C:** NPY-like immunoreactivity is evident in the interstitium surrounding the tubules and ducts. **D:** Substance P labeling is

found in varicosities in the interstitium (arrowheads). Note that the label does not completely surround the secretory tubules. **E:** Dopamine β -hydroxylase immunoreactivity is evident in the wall surrounding blood vessels. No labeling was observed in the interstitium surrounding the secretory tubules. **F:** No immunoreactive label was evident for serotonin in the interstitial space surrounding tubules, ducts or blood vessels. A: bar= 100 μ m; B,C,E,F: bar=50 μ m; D: bar=20 μ m.

Immunofluorescent experiments with the NPY antibody produced similar results (Fig. 2C). A strong fluorescent reaction was evident in the fine nerve fibers and varicosities surrounding the tubules and ducts. In comparison to the VIP experiments, the overall intensity of the fluorescence label with the NPY antibody appeared slightly less than that with the VIP antibody. However, this is only a subjective observation since no quantitative test was performed. No differences in immunoreactivity to VIP and NPY were observed in lachrymal gland tissue of turtles kept in fresh water or in seawater.

The substance P antiserum produced a weak reaction with the fluorescein label (Fig. 2D). The results with anti-SP revealed an intermittent and faint fluorescence running along the base of ducts and occasionally near the secretory tubules. This faint fluorescence was observed in fine nerve fibers running alongside ducts and around some but not all secretory tubules. Substance P-like immunoreactivity was not observed in the adventitia surrounding the blood vessels.

The dopamine β -hydroxylase antibody produced a strong immunofluorescent label in the adventitial area around the blood vessels in the gland (Fig. 2E). Occasionally, some labeled nerve fibers were seen near a secretory tubule or duct, but there was no evidence of these fibers completely surrounding the secretory tubules.

The serotonin antibody showed no immunoreactivity in the interstitial areas surrounding the secretory tubules, ducts, or blood vessels with the fluorescent label (Fig. 2F). All of the histochemical procedures were carried out on turtle brain, as a positive control, to ensure that both the antibody and technique were working. In the mid-brain region, small nerve fibers and varicosities labeled clearly with both the detection methods.

Positive immunoperoxidase labeling, using the polyclonal ChAT antibody, was observed in the rat and turtle brain (Fig. 3A); however, no immunoreactivity for ChAT was evident in the interstitial areas surrounding the secretory tubules, ducts, or blood vessels in the lachrymal gland (Fig. 3B). A dense reaction product was observed in some cells within the tubule in both the experimental and control groups (Fig. 3C). These are suspected of being goblet cells. Pigment granules appear as dark granules clumped closely together and can be distinguished easily from the avidin-biotin reaction product by using light microscopy and through-focusing techniques.

Histochemical localization of the enzyme, ace-

tylcholinesterase, in the turtle brain was used as an indirect assay for acetylcholine. A positive reaction, identified by a dense black stain, appeared in brain cells and faintly in the cellular processes. There was no staining after the omission of acetylcholine or the substitution of acetylthiocholine with butrylthiocholine in the presence of an inhibitor.

In contrast, no acetylcholinesterase staining was observed in the interstitial space surrounding the secretory tubules, ducts, or blood vessels in the lachrymal gland (Fig. 4A). Occasionally, large nerve bundles containing myelinated nerve fibers were identified in the vibratome sections. These were seen running longitudinally through the gland and often in proximity to arterioles. They did not appear to be associated directly with the secretory tissue, and it is not known if the cell bodies of these fibers are located in the central nervous system or in ganglia near or within the lachrymal gland. These nerves did show a dense reaction product in the experimental protocol of the assay (Fig. 4A), but this was absent in the controls (Fig. 4B).

Immunogold labeling

Positive reactions to VIP and NPY antiserum labeled with 14-nm gold particles were observed. Both the VIP-like (Fig. 5A) and NPY-like (Fig. 5B) localization was evident in the axons and nerve terminals. The amount of background label over the noncellular interstitium was very low or negligible compared to the label found within the neurons.

Substance P (Fig. 5C,D) localization occurred in some electron-dense neurosecretory vesicles but not in all of those vesicles contained within a nerve terminal. These were similar in shape and size to the other peptide-containing vesicles. The intermittent identification of substance P in these embedded sections is consistent with the sparse labeling of this peptide in the fluorescence experiments.

Dopamine β -hydroxylase (Fig. 5E) was evident in discrete areas in the neurons but these do not resemble peptide-containing vesicles. Neuronal reactivity was small or limited and restricted to the interstitium near blood vessels of an unknown type.

Double labeling with the VIP and NPY antisera revealed that neurosecretory vesicles in the same neuron reacted positively to both peptides (Fig. 5F). It was not clear if the VIP-like and NPY-like immunoreactivities were co-localized within the same neurosecretory vesicle, but, in some

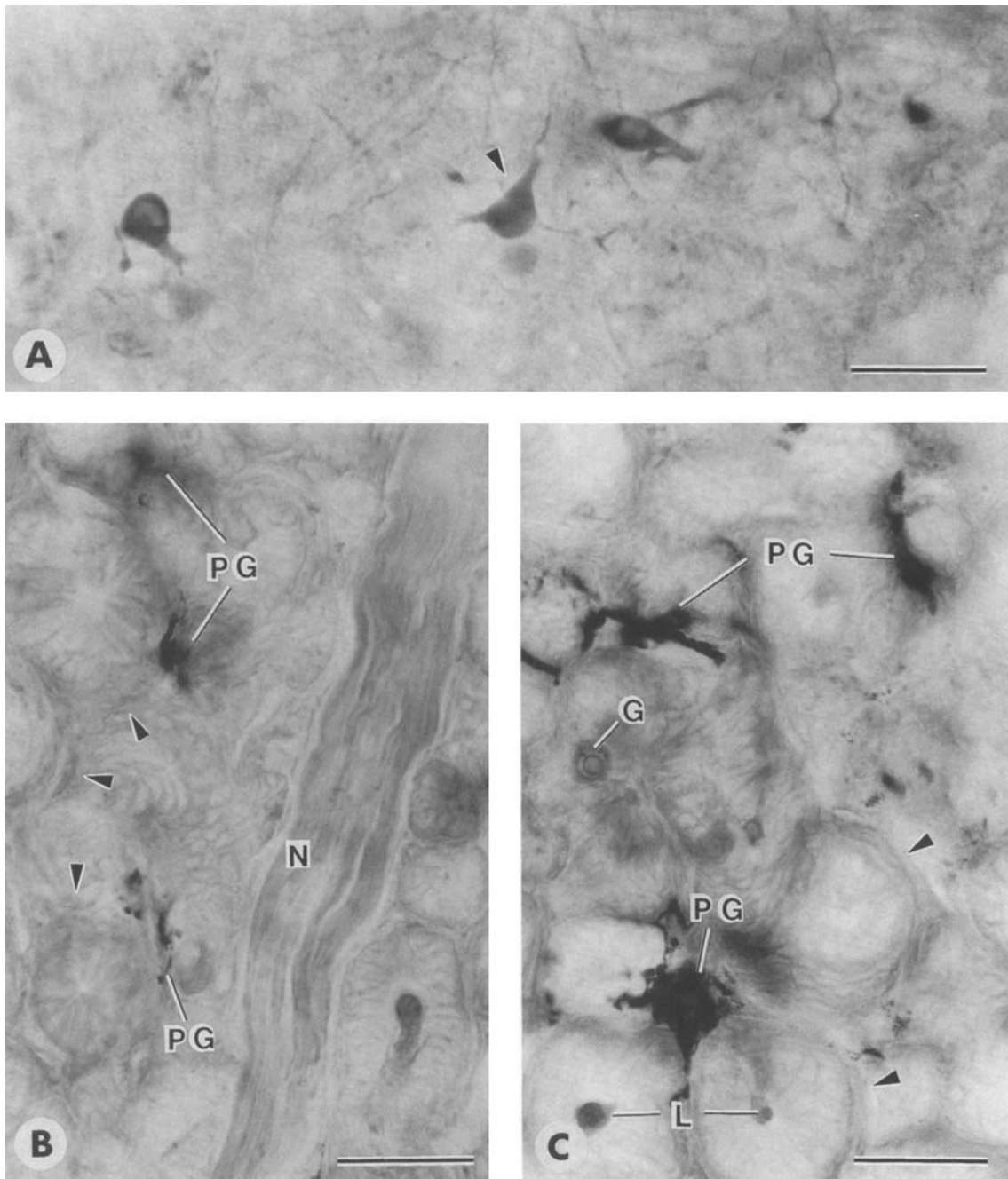


Fig 3. Comparison of immunoperoxidase labeling of ChAT in the turtle brain and lachrymal gland. PG, pigment granules; N, myelinated nerve; L, lumen of tubule; G, goblet-like cell. **A:** Individual nerve cells in the turtle brain labeled positively for ChAT with the avidin-biotin complex (arrowhead). Omission of the primary antibody showed no label in these cells. **B:** Lachrymal gland revealed no staining in the interstitium surrounding the secretory tubules (arrowheads).

Large myelinated nerves traversing the gland also did not reveal any dense avidin-biotin label. Some pigment granules are evident in this preparation. **C:** Omission of the ChAT antibody reveals no discernible difference in the staining characteristics of the lachrymal gland. Pigment granules are evident and non-specific staining is seen in goblet-like cells and in the lumen of the secretory tubules. All figures: bar = 50 μ m.

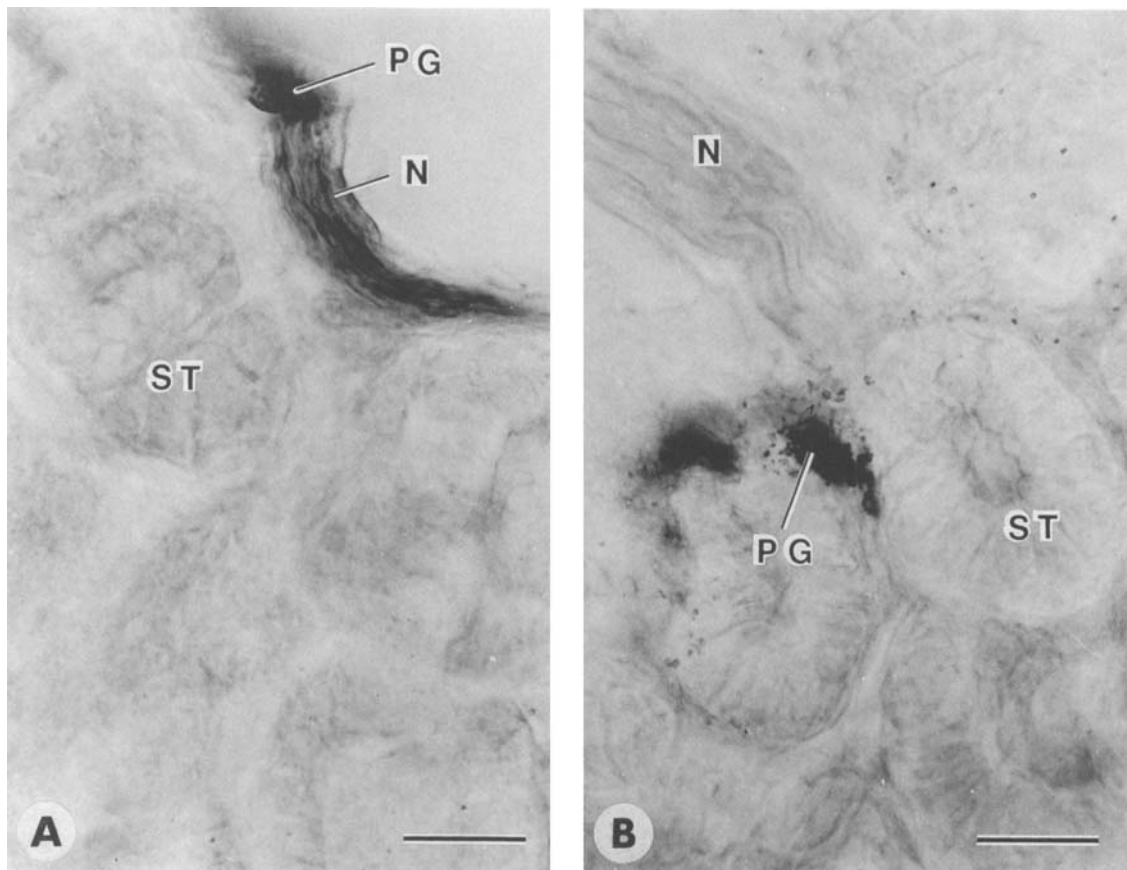


Fig. 4. Acetylcholinesterase localization in lachrymal gland. Large myelinated nerves are acetylcholinesterase positive, but no reactivity was evident in the fine nerve fibers surrounding the secretory tubules. N, nerve; ST, secretory tubule; PG, pigment granules. **A:** Treatment with all reagents

shows a dense label in the large nerve. The wavy appearance of the nerve distinguishes it from pigment granules. **B:** Omission of acetylthiocholine substrate shows no labeling in the large nerve. Dense pigment granules are evident. A,B: bar = 20 μ m.

cases, it appears they were. However, it was evident that these two neuropeptides were localized within the same nerve ending.

DISCUSSION

The innervation of the lachrymal gland in *Malaclemys* was determined to be primarily peptidergic and adrenergic but not cholinergic. The types of neurotransmitters and their location in the lachrymal gland are summarized in Table 1 and are represented diagrammatically in Figure 6. Since the innervation of cranial glands is generally thought to be cholinergic/adrenergic with some possible peptidergic contribution, our observations are significant in their difference from those in most similar studies. In the majority of exocrine glands, including those involved in ion excretion, the major stimulatory neurotransmitter is acetylcholine (Garrett and Kidd, '93; Peaker and Linzell, '75). In some cases there is

also a role for adrenergic nerves (Garrett and Kidd, '93). However, in *Malaclemys*, experiments with a large variety of cholinergic agonists and antagonists have found no evidence of cholinergic control in the lachrymal gland (Cowan, '90). Experiments with adrenergic drugs have indicated a partial stimulation of secretion. The finding that there was strong peptidergic stimulation of the elasmobranch rectal gland (Stoff et al., '79) and the additional observations that peptidergic cotransmitters may be involved in the control of mammalian salivary glands (Garrett and Kidd, '93) suggest that these neuropeptides may be involved in the control of the *Malaclemys* lachrymal gland. Further, some preliminary physiological studies with the close arterial injection of neuropeptides indicated that VIP and substance P promote a strong but short-lived secretion (Cowan, unpublished data). Therefore, the emphasis of this study was placed on the localization of

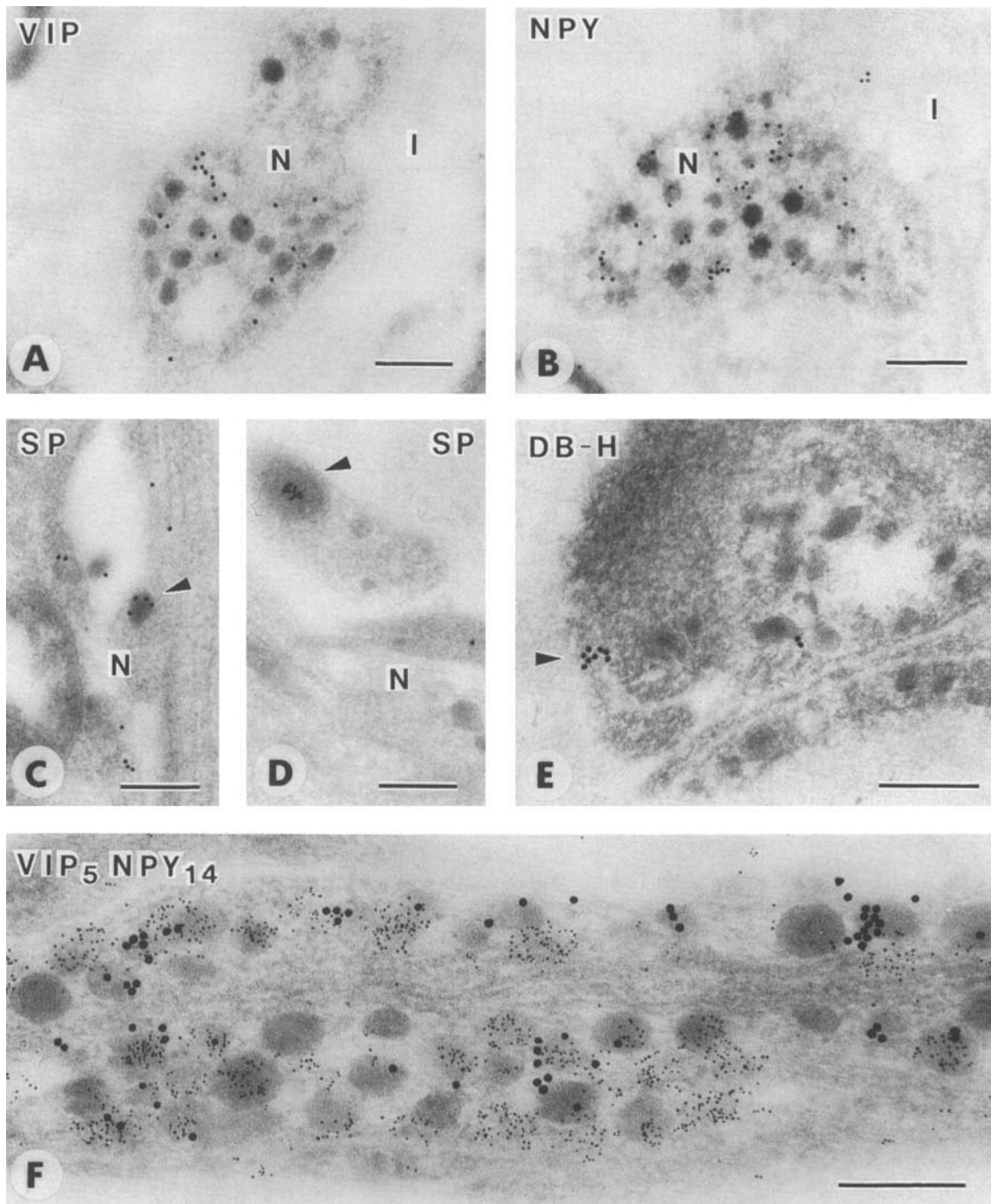


Fig. 5. Immunogold localization of peptidergic and adrenergic agents in the lachrymal gland. N, nerve terminal; I, interstitium. **A:** Nerve terminal in the interstitial space showing dense-cored secretory vesicles labeled with VIP and protein A-gold (14 nm). **B:** Nerve terminals labeled with NPY and protein A-gold. **C,D:** Anti-substance P is labeled in a few

dense-cored vesicles (arrowhead). **E:** Anti-dopamine B-hydroxylase is labeled in discrete areas but not in the dense-cored vesicles containing neuropeptides (arrowhead). **F:** Double-labeling using both sides of the section shows VIP (5 nm gold) and NPY (14 nm gold) are co-localized within the same nerve terminal. All figures: bar = 0.25 μ m.

TABLE 1. Summary of results¹

Neurotransmitters	Label	Results
<u>Immunocytochemistry</u>		
VIP	Fluorescein (n = 4)	+++ acini, ducts
	ABC (n = 1)	+++ acini, ducts
	Gold (n = 4)	+++ acini, ducts
NPY	Fluorescein (n = 4)	+++ acini, ducts
	ABC (n = 1)	+++ acini, ducts, blood vessels
	Gold (n = 4)	+++ acini, ducts
Substance P	Fluorescein (n = 4)	+ acini, ducts
	(Gold (n = 2))	+ around acini
D β -H	Fluorescein (n = 4)	+++ blood vessels, ducts
	ABC (n = 1)	+++ blood vessels, ducts
	Gold (n = 4)	+
Serotonin	Fluorescein (n = 4)	0
	ABC (n = 1)	0
	Gold (n = 4)	+++brain
ChAT	ABC (n = 3)	0 acini, ducts, blood vessels ± large nerve +++ brain
<u>Histochemistry</u>		
Acetylcholine	Acetylcholinesterase (n = 3)	++ large nerve +++ brain 0 acini, ducts, blood vessels

¹Code: n, No. of animals; 0, none; ±, intermittent or not conclusive; +, weak; +++, strong.

possible controlling factors involved in the lachrymal gland secretion by immunohistochemical techniques.

Peptidergic innervation

Both VIP and NPY antisera, with the fluorescent label, bound to nerve fibers within the interstitial space surrounding the tubules and ducts. This was confirmed by the immunogold labeling technique. The appropriate control procedures, performed in both immunolabeling methods, supported the specificity of the results. Therefore, the evidence indicates there is a VIP-like and NPY-like immunoreactivity in the nerves surrounding the secretory tubules and ducts in the lachrymal gland. These results are consistent with studies of other exocrine glands. VIP and NPY have been demonstrated in the mammalian salivary glands, submandibular and parotid, and in mammalian lachrymal and nasal glands (Dartt et al., '88; Yokoyama et al., '91). However, VIP is usually found co-existing with cholinergic neurons, while NPY has been reported in noradrenergic neurons (Lundberg and Hokfelt, '86). The co-existence of VIP and NPY has been described in parasympathetic and sympathetic neurons and, in particular, has been demonstrated in adrenergic nerve fibers in tracheal smooth muscle of the guinea pig

(Gibbins and Morris, '88). NPY is a potent vasoconstrictor, and its presence alongside catecholamines has created much interest in its role in innervating vascular smooth muscle. In general, NPY shows greatest density in the adventitial area of the muscular arteries (Polak and Bloom, '88). Since adrenergic neurons were not found surrounding the secretory tubules, the area of greatest NPY innervation in this study, it is unlikely these two neurotransmitters co-exist here. Nor could the co-existence of NPY and noradrenaline in neurons surrounding arterioles be demonstrated with the immunogold method in this study. With respect to osmoregulating glands, VIP and NPY have been also shown in the avian nasal gland (Gerstberger, '91), although it is known to be controlled mainly by cholinergic neurons, and VIP exists in the rectal gland of elasmobranchs (Stoff et al., '79; Shuttleworth and Thorndyke, '84; Silva et al., '87).

Substance P produced a weak, localized immunofluorescence in the interstitial space surrounding the ducts. No label was observed surrounding the secretory tubules or blood vessels. This peptide is known to co-exist with conventional neurotransmitters in efferent nerves. It is also found in some sensory afferent neurons associated with the ducts in the rat submandibular gland (Goedert

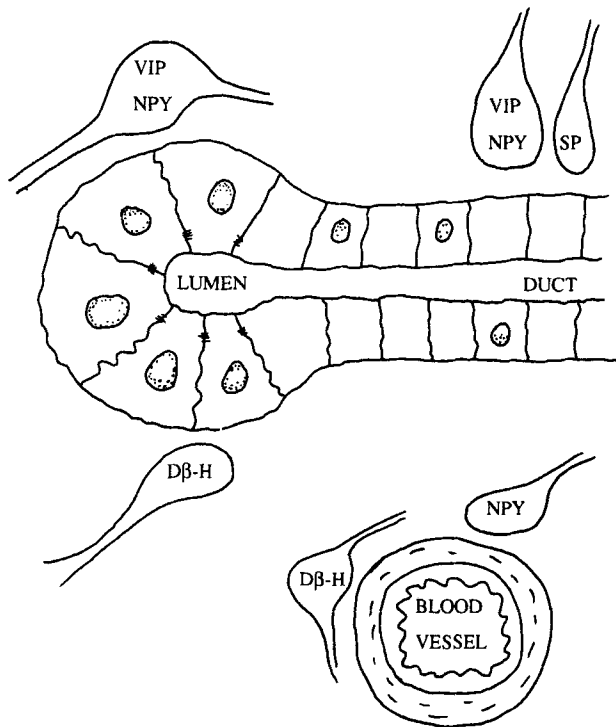


Fig. 6. Diagrammatic summary of adrenergic and peptidergic innervation in the lachrymal gland. The co-existence of VIP-like and NPY-like immunoreactivity was observed in nerves surrounding the secretory tubules and ducts in the lachrymal gland. Occasionally NPY-like immunoreactivity was seen in the outer wall of blood vessels. Substance P-like immunoreactivity was seen in the interstitium near ducts. No label was observed surrounding the secretory tubules or blood vessels. The synthesizing enzyme for norepinephrine and epinephrine, dopamine β -hydroxylase, was found primarily in the outer wall of the blood vessels and occasionally in the interstitium of secretory tubules. No label was evident for choline acetyltransferase the synthesizing enzyme for acetylcholine, or for the degrading enzyme of acetylcholine, acetylcholinesterase. No immunoreactivity was observed for serotonin.

et al., '82). Other studies in the rat salivary glands show the co-existence of substance P and calcitonin gene-related peptide in afferent neurons, and these peptides have been associated with the larger ducts and blood vessels (Ekstrom et al., '88). In the lachrymal gland, it is not known what the function of substance P is nor in what type of nerve it is found. As a neuromodulator, it may facilitate or inhibit other neural functions. Its presence surrounding the ducts could suggest some part in altering the osmolarity of the secretion. There is evidence that plasma proteins and other relatively large molecules pass from the blood into the duct system (Coghlan, '82). This would indicate a high permeability of parts of the tubular

epithelium. As the final secretion is very hyperosmotic, the terminal portion of the tubules may serve to concentrate the final secretion by reabsorption of water and/or secretion of additional sodium. Substance P may be involved in the control of either of these putative processes. However, substance P could also be associated with afferent sensory nerves, in which case it may respond to noxious stimuli by facilitating secretions. Substance P has been linked with the secretion of IgA and IgG immunoglobulins in secretions of mammalian lachrymal tears (Dartt, '89). This is consistent with immunoglobulins found in the lachrymal gland tears of *Malaclemys* (Cowan, unpublished data).

Adrenergic innervation

The synthesizing enzyme for norepinephrine and epinephrine, dopamine β -hydroxylase, was found in the adventitial layer of the blood vessels, particularly arterioles, and occasionally in the interstitium of ducts or tubules. Adrenergic neurons did not appear to innervate the secretory tubules or ducts directly. Norepinephrine and/or epinephrine could be vasodilators, and the effect of this on blood flow is consistent with the sporadic secretions obtained from the injections of these neurotransmitters in physiological experiments (Cowan, unpublished data) and also in the avian salt gland. In the latter study, Gerstberger ('91) found control of blood flow is an important means of controlling salt secretion in salt-water-adapted ducks. This represents a slightly different pattern than in mammalian salivary glands in which adrenergic neurons are found directly innervating the secretory tubules.

Cholinergic innervation

Both acetylcholinesterase histochemistry and ChAT immunolabeling were observed in the rat and turtle brain. In the lachrymal gland, no label was observed in the small nerves or nerve endings surrounding the secretory tubules, ducts, or blood vessels with either of the labeling techniques. These findings are consistent with those from physiological studies indicating that stimulatory control is not cholinergic. The apparent lack of cholinergic innervation in an exocrine gland is unusual since acetylcholine is a major controlling neurotransmitter in most cranial glands in vertebrate species. One possible reason may be related to the tonicity of the secreted fluid. In glands which are controlled by cholinergic neurons, the secretions tend to be copious and di-

lute, whereas in the *Malaclemys* lachrymal gland, as in other salt glands, the secretions tend to be hypertonic.

Furthermore, it is suspected that the lachrymal gland in *Malaclemys* is not a dedicated salt gland but may have additional functions other than ion regulation (Cowan, '90). Physiological experiments indicate that the actual amount of sodium chloride secreted varies under identical conditions and that salt-loading does not always evoke a secretion. In addition, it was found that irritating fumes, such as cyanoacrylate glues, used in affixing eyecups to collect secretions, produced highly concentrated tears from the lachrymal gland. It is suggested that since both ionic and non-ionic stimuli produce a similar secretory response, the lachrymal gland in this animal may serve more than one role (Cowan, '90).

The unique neurotransmitter profile in the lachrymal gland may be related to the organism being euryhaline. Non-osmoregulating glands produce secretions that contain large volumes of water and variable amounts of proteinaceous material which is normally controlled by cholinergic and adrenergic innervation. In contrast, the role of the lachrymal gland in *Malaclemys terrapin* is thought not to be primarily the secretion of water but its conservation. The organic component of gland secretions is perhaps not important. It appears that in glands where acetylcholine is present, large volumes of secretion are produced, whether it be hypertonic, as in other salt glands, or isotonic, as in mammalian salivary glands. In this case, in the lachrymal gland of *Malaclemys*, the volume of secretion is low and sodium concentration is high, perhaps because there is no cholinergic innervation. The role of this gland may be the primary secretion of ions or the secondary reabsorption of water in the duct, and perhaps the high concentration of peptides found surrounding the tubules and ducts aids the gland in serving that role. Also, cholinergic innervation in salivary glands is associated closely with myoepithelial cells and nerves within the basement membrane abutting onto the secretory cells. In the lachrymal gland, myoepithelial cells do not exist, and in this study no nerves were found inside the basement membrane. Again, this may reflect the lack of any need for cholinergic innervation in this osmoregulating gland. The extent of innervation of VIP and NPY surrounding the secretory tubules raises interesting questions into its primary role in the control of secretions in this gland.

ACKNOWLEDGMENTS

We thank Bob Crowther for his valuable technical advice throughout this study and Roger Smith for his photographic assistance. Financial support for this study was provided by the University of New Brunswick through a Research Fund and a Futures Fund to Dr. F.B.M. Cowan and by NSERC through an Infrastructure Grant to the Electron Microscopy Unit, University of New Brunswick.

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