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Ultrastructure and lectin cytochemistry of secretory cells in lingual glands of the Japanese quail (*Coturnix coturnix japonica*)

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Summary. In the present study, as continuation of our previous research, Japanese quail (*Coturnix coturnix japonica*) lingual glands were investigated by means of transmission electron microscopy (TEM) to understand the cytoarchitecture and the subcellular sugar distribution within the different secretory structures. Indeed, glycosidic residues were visualized by applying an indirect technique of binding and the terminal sialoglycoconjugate sequences were characterized by employing sialidase digestion combined with lectin affinity. The ultrastructural analysis revealed an unusual cytoarchitecture of the caudal portion of anterior lingual gland that was composed of both secretory cells, filled with granules, and non-secretory cells, filled with mitochondria. Conversely, the posterior lingual gland was composed of secretory units of lingual glands only containing mucous cells filled with secretory granules with a variable morphology, including bipartite features characterized by an electron-lucent matrix and one or more electron-dense areas. Actual findings further supported that the quail lingual glands produce sialoglycoconjugates characterized by a heterogeneous composition. In conclusion, the cytological characteristics and the carbohydrate composition of quail lingual glands suggest that, analogously to mammal salivary glands, avian lingual glands could also be involved in several functions that can be correlated with the occurrence of sialic acids.

Key words: TEM, Lectin cytochemistry, Quail, Lingual glands

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Introduction

Saliva is a complex liquid consisting of secretions from the major and minor salivary glands (Lingström and Moynihan, 2003).

Avian salivary glands produce a large amount of glycoconjugates (Gargiulo et al., 1991; Menghi et al., 1992) but compared to the corresponding macromolecules of mammals, show differences in sugar moieties in the core region of the carbohydrate chains (Scocco et al., 1995). A morphofunctional characterization of salivary glands of chicken in relation to particular feeding habits suggested that salivary gland products are involved in basic functions, as has been previously described for other animals, showing overlapping histochemical staining patterns (Samar et al., 2002).

Light and confocal analyses were previously performed by us on the quail lingual glands by conventional carbohydrate staining methods and lectin histochemistry combined with specific exoglycosidase digestion; these approaches were used to investigate the histology of these glands and to visualize the occurrence and spatial distribution of salivary gland glycoconjugates (Menghi et al., 1993). The quail lingual glands were found to be composed of both anterior and posterior lingual glands with the anterior lingual gland divided in rostral and caudal portions. In particular, the rostral portion only secreted neutral glycocomponents in contrast to the caudal portion, while the posterior lingual glands were found to be extremely heterogeneous and produced acidic glycoconjugates. The glycosidic components of the quail lingual glands were found to be heterogeneously distributed within secretory units of lingual glands, as well as within each secretory cell (Bondi et al., 2000).

Considering that the study of lingual glands in birds is a rare subject of research, and since salivary glands

are a good model to investigate the relationship between cell secretion and glandular structure, the aim of the present study is to complement the knowledge relating ultrastructure and histochemistry in granivours birds. In particular, the present investigation extended our previous data on Japanese quail (*Coturnix coturnix japonica*) lingual glands by means of transmission electron microscopy (TEM) to understand the cytoarchitecture and the subcellular sugar distribution within different secretory structures. Indeed, the glycosidic residues were visualized by applying an indirect technique of binding, based on lectin-horseradish peroxidase conjugates, antiperoxidase antibody, and protein A-gold (Menghi et al., 1996); moreover, the terminal sialoglycoconjugate sequences were characterized by employing sialidase digestion combined with lectin affinity to the subterminal acceptor sugars.

Materials and methods

Reagents

The lectin panel was selected on the basis of glyconjugates composition, which usually exhibits mannose (α-Man) in internal position, N-acetyl-glucosamine (ß-GlcNAc) and N-acetylgalactosamine (α-D-GalNAc) in O- and N- glycosidic bonds, as well as in subterminal position, sialic acid in terminal position with galactose (α-D-Gal) and α-D-GalNAc as acceptor sugars. Table 1 shows the lectins used and their sugar-binding specificity. All horseradish peroxidase (HRP)-labelled lectins, anti-horseradish peroxidase antibody (raised in rabbit), protein A-gold (10 nm) and reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Durcupan ACM (Araldite base embedding agent for electron microscopy) and Bioacryl resins were purchased from Fluka (Basel, Switzerland) and Bio-Optica (Milano-Italy), respectively.

Tissue samples

Ten quails, *Coturnix coturnix japonica*, two months old, were killed according to the recommendation of the Italian Ethical Committee. Animals, with free access to water and pellet food until removal of tissue, were anaesthetized with ether and killed by decapitation.

Table 1. Lectins used in the present ultrastructural study.

Source of lectin	Abbreviation	Carbohydrate binding specificity
Dolichos biflorus	DBA	α-D-GalNAc
Arachis hypogaea	PNA	ß-D-Gal-[1-3]-D-GalNAc
Triticum vulgaris	WGA	ß-GlcNAc>>sialic acid residues
Canavalia ensiformis	Con A	α-D-Man>α-D-Glc
Erytrina cristagalli	ECA	ß-D-Gal-[1-4]-D-GlcNAc

Anterior and posterior tongue portions were removed immediately after sacrifice and processed for ultrastructural analysis and lectin cytochemistry.

Ultrastructural analysis

The quails' tongues were quickly dissected and placed in cold fixative solution. Then, samples were cut into small pieces (~ 1 mm^3) and immersed in fixative solution. Eight/ten pieces from each tongue were subsequently processed for routine electron microscopy. They were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h at room temperature and post-fixed in 1% osmium tetroxide in 0.1 M veronal acetate buffer, pH 7.4, for 2 h at 4°C. After washing, samples were dehydrated and embedded in Durcupan ACM. Ultrathin sections (about 60 nm thick) were stained with uranyl acetate (10 min at 30°C) and lead citrate (1 min at 20°C) by an LKB Ultrostainer.

Lectin cytochemistry

For ultrastructural lectin cytochemistry, tissue samples were processed as described above and fixed in a solution containing 4% paraformaldehyde, 1% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.6, in presence of 0.5 mM $CaCl_2$ for 3 h, at 4°C to better preserve the glucidic components packaged in secretory granules. After quenching of free aldehydes with 50 mM ammonium chloride in sucrose-phosphate buffer for 1 h at 4°C, samples were treated with 0.1 M maleate buffer, pH 6.5, added with 3.5% sucrose 4 times, 15 min each at 4°C and post-fixed in 1% uranyl acetate in sucrose-maleate buffer, final pH 6.0, for 2 h at 4°C.

The dehydration step was performed twice, 5 min each time, with acetone at increasing concentrations (50%, 70%, 90%), at 4°C and under gentle stirring.

The embedding step was carried out in Bioacryl resin. The specimens were infiltrated in a solution of 100% Bioacryl (twice, 2 h each time) at 4°C under gentle stirring and subsequently immersed overnight in fresh pure resin at 4°C; the polymerization was performed by UV light for 72 h at 4°C in carefully closed gelatine embedding capsules filled up with resin and degassed for 30 min at 4°C.

The indirect lectin binding was applied to freshly cut thin sections (about 60 nm thick) mounted on uncoated, 400 mesh nickel grids (Menghi et al., 1996). HRP-labelled lectins were used under the following conditions: DBA, PNA, WGA, Con A, ECA (2 µg/ml) were diluted in 0.1 M Tris buffered saline (TBS), pH 7.4, plus 1% bovine serum albumin (BSA). After lectin incubation, prolonged for 3 h at room temperature, sections were rinsed with 0.05 M TBS containing 1% BSA and reacted with anti-HRP antibody (diluted 1:100 in TBS-1% BSA) for 1 h at room temperature. Finally, sections were washed with 0.05M TBS-1% BSA and treated with protein A-gold (10 nm) diluted 1:50 in 0.1

M TBS, pH 7.4, plus 1% BSA and 0.05% Tween 20, for 1 h at room temperature. After counterstaining, with uranyl acetate (10 min at 30°C) and lead citrate (1 min at 20°C) by an LKB Ultrostainer, samples were carbon-coated and analyzed in a Philips 201C TEM.

Sialidase digestion

Sections were incubated with sialidase (neuraminidase type V, from *Clostridium perfringens*; Sigma Aldrich, Milano, Italy) at a concentration of 0.5 U/ml in acetate buffer 0.1 M, pH 5.5, containing 10 mM $CaCl_2$ for 16 h at 37°C (Menghi et al., 1998).

Controls

Controls for the specificity of the lectin binding were run with sections prepared by incubating the lectin solutions with their specific competing sugars (N-acetyl-D-galactosamine for DBA, D-galactose for PNA and ECA, N-acetyl-D-glucosamine for WGA, D-mannose for Con A) at concentrations of 0.2-0.4 M. Additional controls provided the omission of the antiperoxidase antibody or lectin-HRP conjugates. Controls for sialidase digestion aimed to investigate both the action of the enzyme-free buffer and the specificity of the enzymatic treatment.

Results

Morphology

Anterior lingual gland

The quail lingual glands comprise the anterior lingual gland, with rostral and caudal portions that differ in morphology, as well as histochemical staining, and the posterior lingual gland devoid of regional differences. The rostral portion was composed of a homogeneous cell population containing a few secretory granules

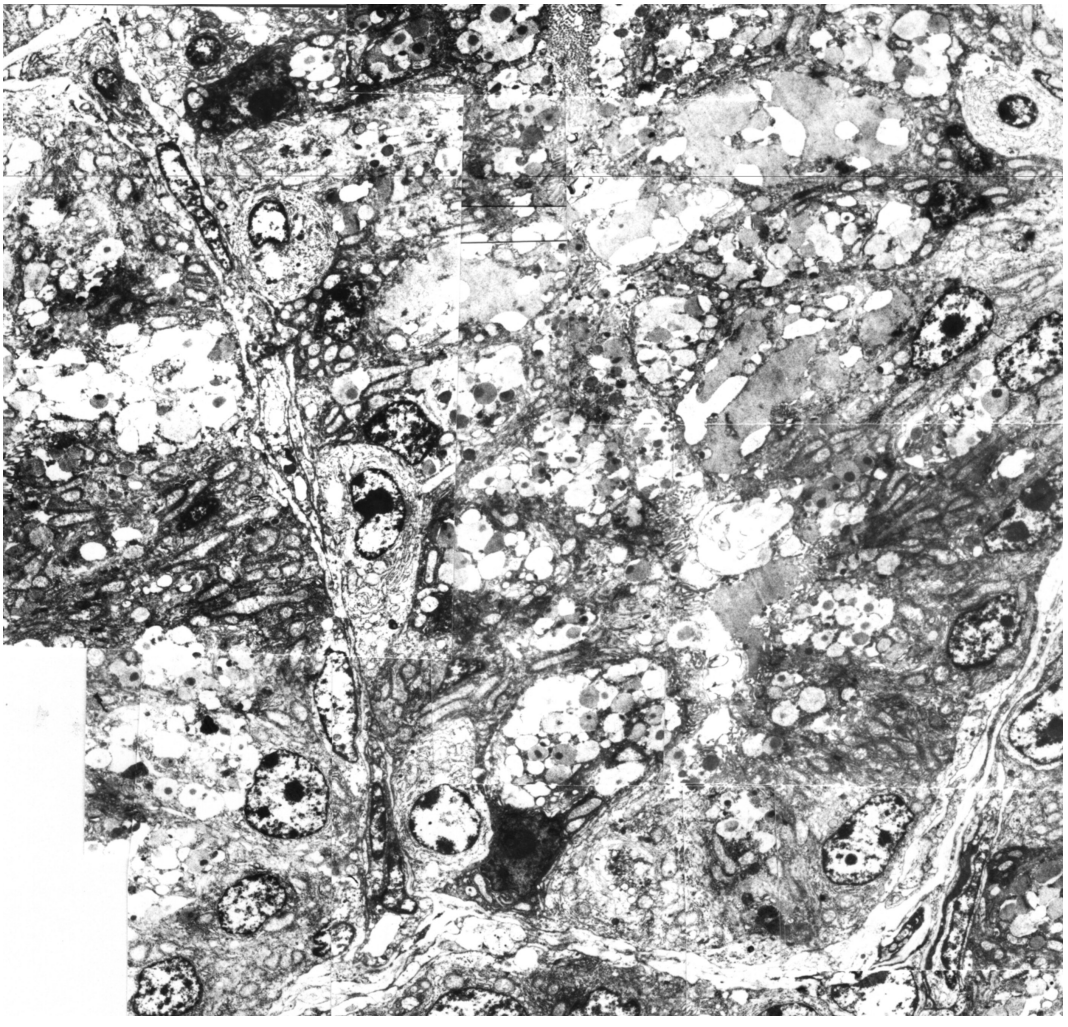

Fig. 1. Electron micrograph reconstruction of caudal portion of the anterior lingual gland. This portion was arranged in tubulo-acini and in tubules. Within tubules secretory and non-secretory cell population both surrounding a lumen were found. x 9,240

surrounding a wide lumens.

In contrast to the rostral portion organized in secretory tubules (data not shown), the caudal portion was arranged partly in tubulo-acini and partly in tubules composed of specialized cell populations. Within tubules two cell types were clearly distinguished: a great majority of secretory cells with a lot of granules, and a few intercalated non-secretory cells with a high content of mitochondria. Both secretory and mitochondria-rich cells surround a lumen where secretory products are exocyted (Fig. 1). Secretory cells showed, at the supranuclear region, numerous bipartite, as well as more or less electron-lucent, secretory granules and, in the basal region, a nucleus with dispersed euchromatin and an evident nucleolus with associated heterochromatin (Fig. 2).

Posterior lingual gland

The posterior lingual gland appeared to be composed of secretory units only containing cells of columnar shape (Fig. 3A).

The tubules were found to be formed by secretory cells filled with unmerged and merged secretory granules with electron-transparent matrix and eccentric dense spherules. The nucleus was located on the basal region; intercellular tissue spaces were observed between adjacent cells delimiting the luminal border with short microvilli. Among secretory granules belonging to the same cell, some merged granules protruding into the lumen were observed (Fig. 3B).

The secretory granules varied in size and most of them showed bi- or tripartite structures, with an electron-lucent matrix and electron-dense peripheral areas. Some granules showed an electron-dense cap (Fig. 3C).

Lectin cytochemistry

DBA

The rostral portion of the anterior lingual gland did not appreciably react to DBA lectin. Conversely, the caudal portion showed heterogeneous DBA binding within cells of the same tubulo-acini. Gold particles were found to be located in restricted areas (Fig. 4A) of secretory granules as well as in the lumen (Fig. 4B). Sialidase digestion increased DBA affinity (data not shown).

In the posterior lingual gland, DBA showed heterogeneously distributed gold particles which largely differed in number between adjacent secretory cells (Fig. 4C). In particular, the heterogeneous secretory granules showed DBA labeling above all in the electron-lucent matrix (Fig. 4D); the affinity of the electron-lucent granules was greatly enhanced by sialidase digestion (Fig. 4E).

ECA, PNA

In the anterior caudal lingual gland, ECA (Fig. 5A) and PNA (Fig. 5C) lectins showed some positive sites more numerous in the electron-lucent than electron-dense secretory materials. Sialidase digestion induced numerous new ECA (Fig. 5D) and PNA (Fig. 5B) reactive sites. In the posterior gland only sialidase/PNA staining showed affinity sites (data not shown). The control for indirect binding entailed the omission of PNA-HRP conjugate (Fig. 5E).

WGA, ConA

WGA lectin showed a modest affinity indicated by scattered gold signals in the anterior caudal lingual gland (Fig. 6A). In the posterior lingual gland, many gold particles demonstrating WGA occurrence were found

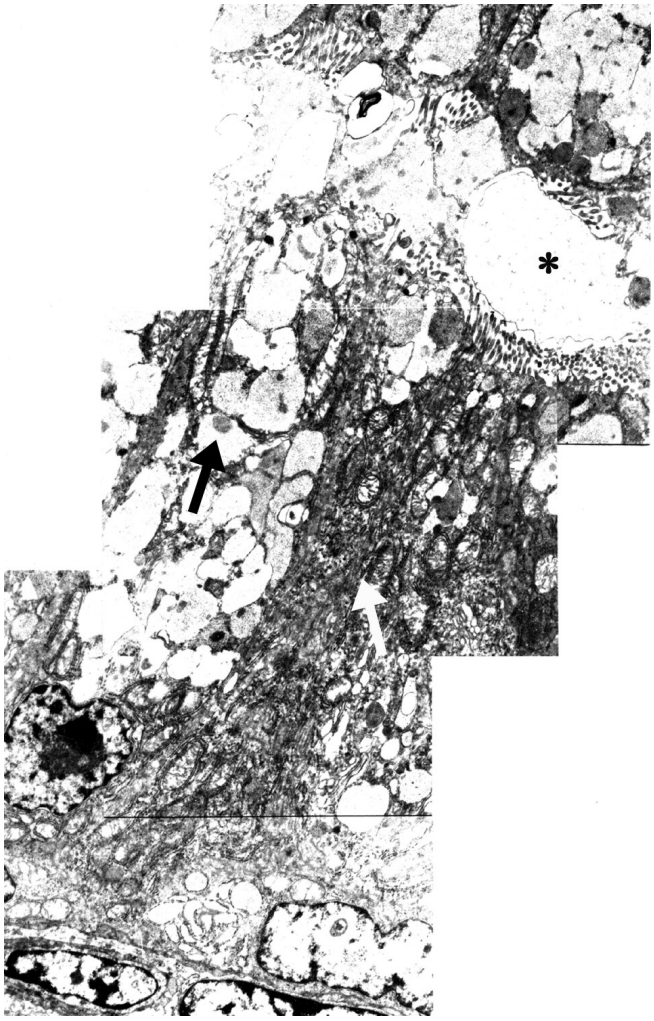

Fig. 2. Electron micrograph reconstruction of caudal portion of the anterior lingual gland. A higher magnification better illustrated the occurrence of two types of cells: secretory cells (black arrow) and mitochondria-rich cells (white arrow) with apical microvilli longer than those of secretory cells. The former is filled with secretory granules; the latter presents a high content of elongated and round shaped mitochondria. The lumen is marked by an asterisk. x 13,200

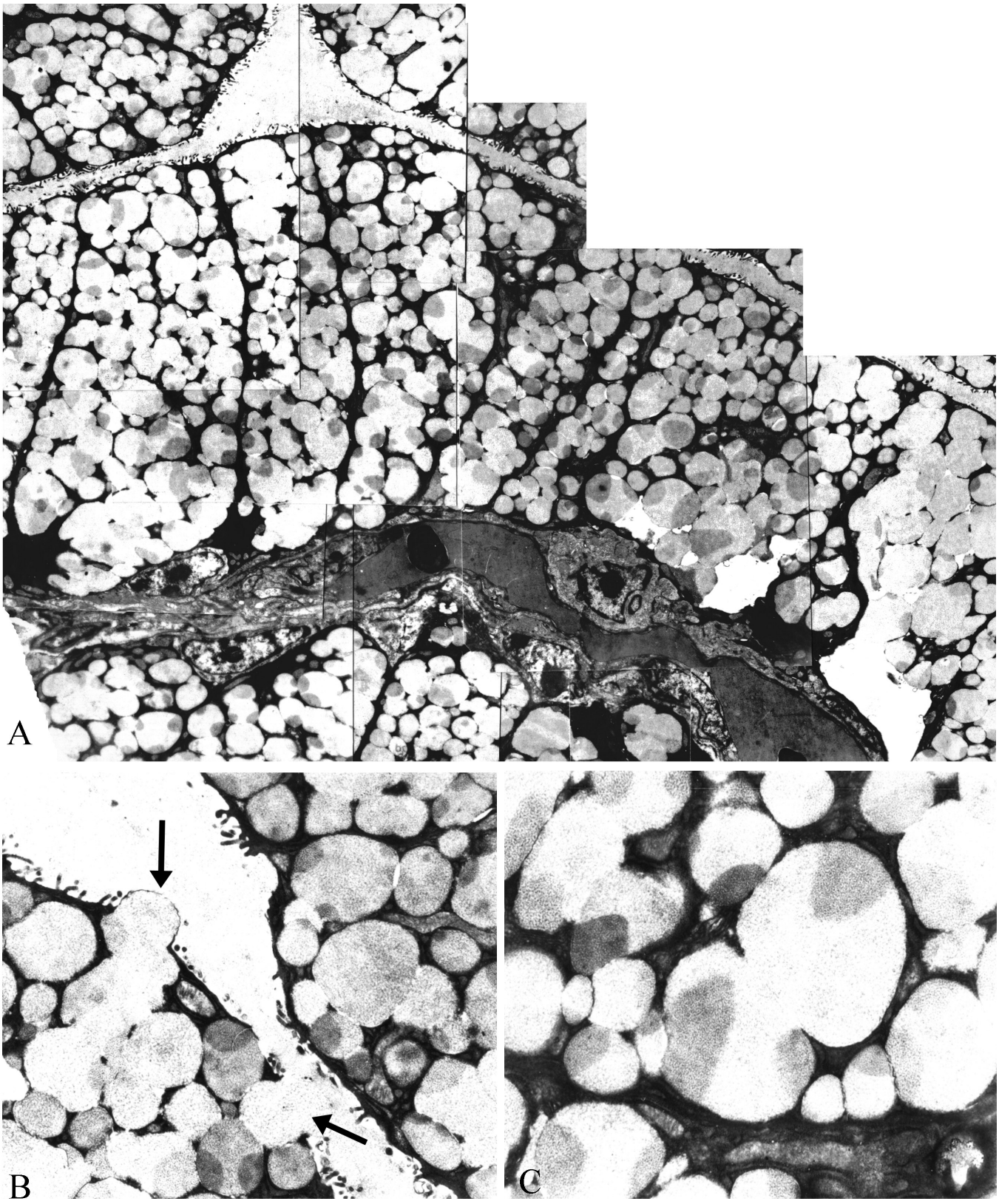

Fig. 3. A. Electron micrograph reconstruction of the posterior lingual gland. Secretory cells with columnar shape were organized as tubules. **B.** Ultrastructural micrographs of the posterior lingual gland. Note the presence of short microvilli surrounding the lumen. Some secretory granules are protruding into the lumen (arrows). **C.** Most of the granules showed a bipartite structure with characteristic electron-dense spherules dispersed in electron-transparent matrix. A, x 6,100; B, x 14,200; C, x 26,100

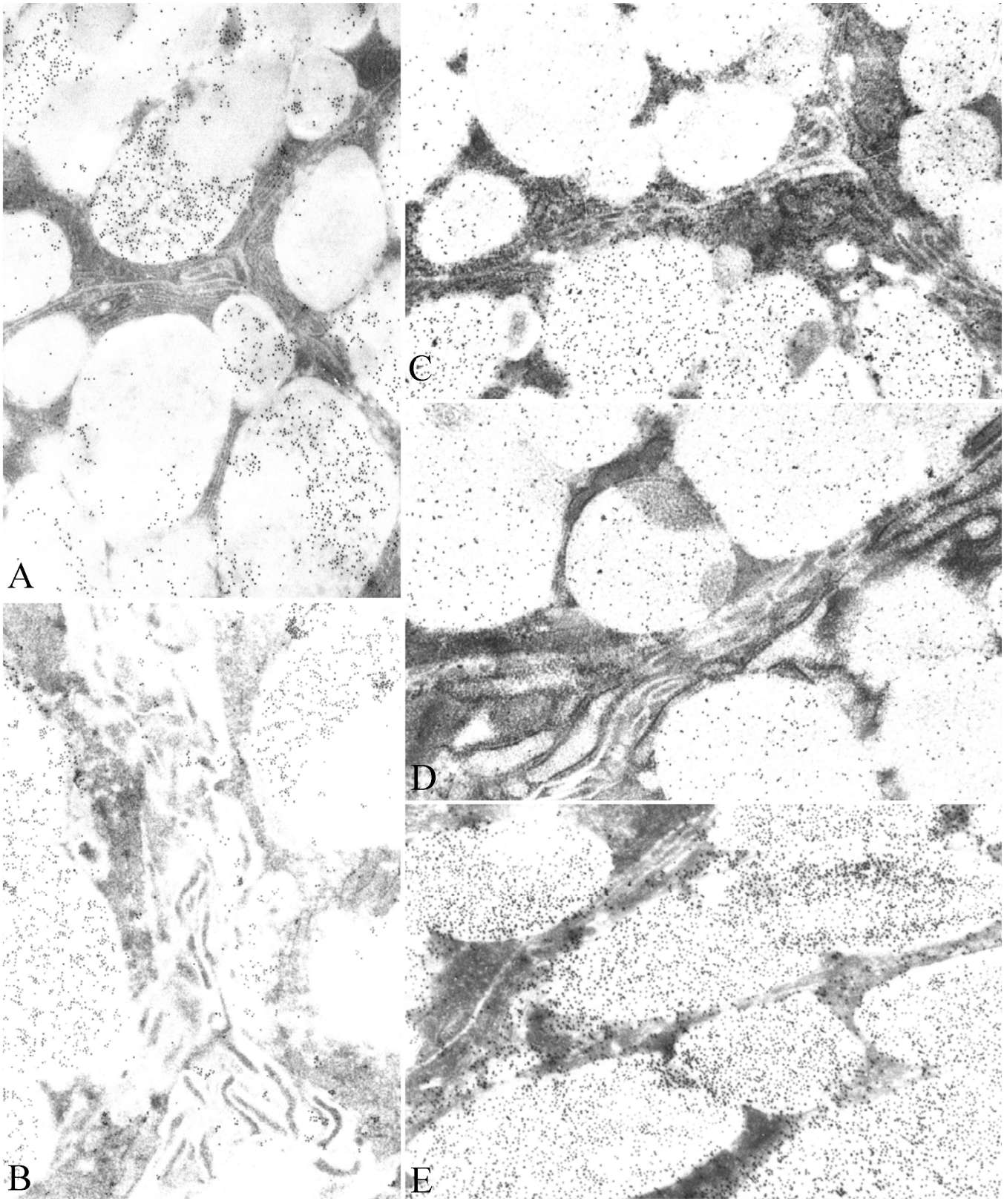

Fig. 4. DBA staining. **A, B.** Anterior lingual gland. Caudal portion. Electron-lucent granules showed affinity binding sites reflecting the presence of terminal N-acetyl-D-galactosamine; also, the lumen contained positive DBA binding sites. **C, D.** Posterior lingual gland. Gold particles were found to be heterogeneously distributed on the electron-lucent matrix. **E.** A strong increase of DBA affinity occurred after sialidase digestion. A, E, x 30,500; C, D, x 26,400; B, x 24,000

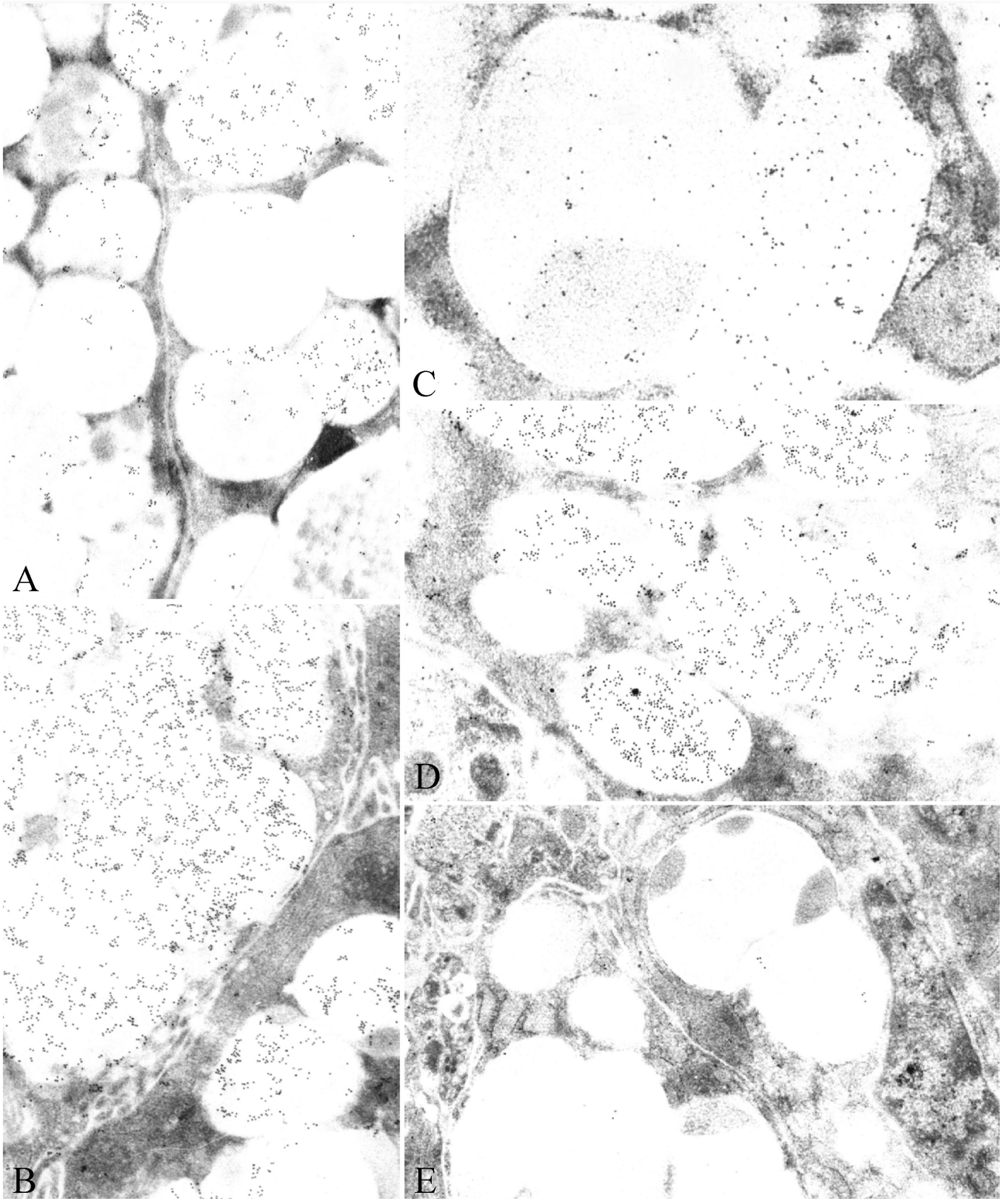

Fig. 5. ECA and PNA staining. Anterior lingual gland. Caudal portion. **A, C.** ECA and PNA gold particles indicated modest binding at secretory granule level. **D, B.** After removal of terminal sialic acid new reactive sites were observed for both lectins. **E.** Control for indirect binding. A, E, x 24,500; B, x 25,500; C, D, x 33,500

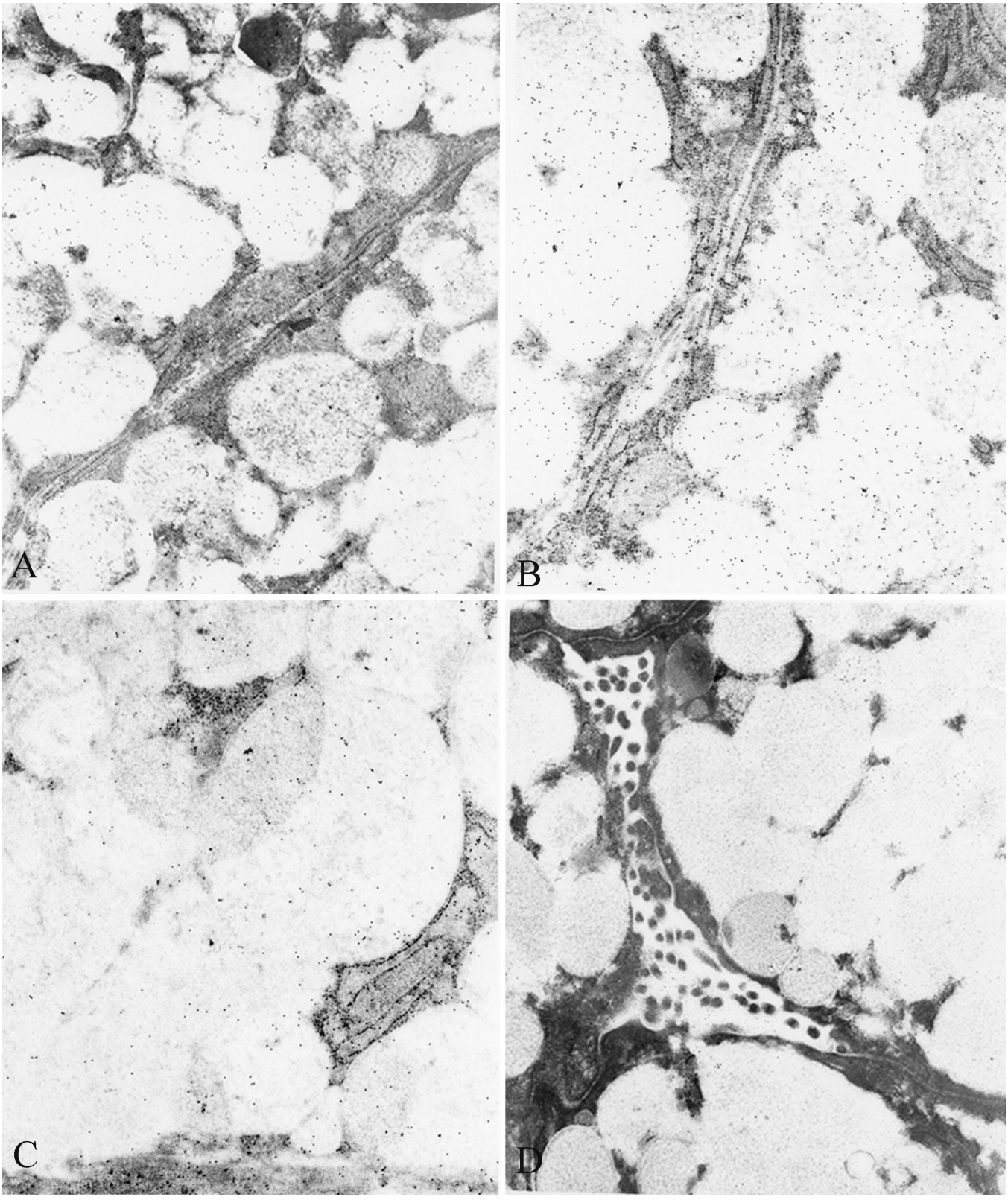

Fig. 6. WGA binding. **A.** Anterior lingual gland. Caudal portion. Modest labeling was evidenced. **B.** Posterior lingual gland. Note the moderate binding on electron-lucent granules. **C.** Con A staining. Anterior lingual gland. Caudal portion. Only a few reactive sites were observed. **D.** In control section Con A staining was not appreciable. A, D, x 23,200; B, C, x 26,400

Table 2. Lectin cytochemistry results.

Lectin	Main binding sugar specificity	Gland/Secretory Granule		
		Anterior, rostral	Anterior, caudal	Posterior
DBA (*Dolichos biflorus*)	α-D-GalNAc	+/-	+	+
Sial/DBA	sialic acid linked to α-D-GalNAc	+/-	++	+++
PNA (*Arachis hypogaea*)	ß-D-Gal-[1-3]-D-GalNAc	+/-	+/-	+/-
Sial/PNA	sialic acid linked to ß-D-Gal-[1-3]-D-GalNAc	+/-	++	++
ECA (*Erythrina cristagalli*)	ß-D-Gal-[1-4]-D-GlcNAc	+/-	+/-	-
Sial/ECA	sialic acid linked to ß-D-Gal-[1-4]-D GlcNAc	+/-	++	-
WGA (*Triticum vulgaris*)	ß-GlcNAc>>NeuNAc	+/-	+/-	++
ConA (*Canavalia ensiformis*)	α-Man>α-Glc	+	+/-	-

Results are given in arbitrary units as follow: 0, negative; +/-, weak; +, moderate; ++ strong; +++, very strong binding.

(Fig. 6B). As concerns Con A lectin, a few reactive sites were observed on secretory granules (Fig. 6C). Con A control section did not show gold particles on either secretory granules or lumen (Fig. 6D).

Results for lectin cytochemistry were summarized in Table 2.

Discussion

In the present study we analyzed the morphology and subcellular distribution of sugars at TEM level by applying conventional and indirect technique of lectin binding combined with sialidase digestion. The colloidal gold technique using lectins labeled to both gold particles and horseradish peroxidase, on ultrathin sections embedded in the acrylic hydrophilic resins Bioacryl, was shown to be the most satisfactory technique to visualize individual sugar residues on secretory tissues (Menghi et al., 1996).

Quail lingual glands consist of anterior and posterior glands. In the anterior gland, two distinct portions were identified, a rostral portion and a caudal portion consisting of tubulo-acini and tubules. The posterior lingual gland is a tubuloacinar gland that produces mucous material (Menghi et al., 1993). Liman et al. (2001) reported a different anatomical nomenclature for salivary glands of the quail tongue.

Actual results extended findings originated from previous optical and confocal studies performed on the quail (*Coturnix japonica*) lingual glands (Menghi et al., 1993; Bondi et al., 2000); indeed, the TEM approach gave additional and complementary information about the ultrastructural morphology and the occurrence of sialoglycoconjugates within each secretory granule. In particular, the ultrastructural analysis revealed an unusual cytoarchitecture of the anterior lingual gland caudal portion that was composed of both secretory cells, filled with granules, and non-secretory cells, filled with mitochondria. A similar organization of secretory and mitochondria-rich cells was found in the angularis oris salivary gland of the house sparrow (Nagato and Tandler, 1986). Due to the lack of ductal segments, the intercalated mitochondria-rich cells can participate in the homeostasis by modifying the final saliva composition; accordingly, these cells exhibit the classical ultrastructure of absorptive cells. Dark cells with a high content of mitochondria and pale cells with a low content were only observed in the striated duct of hare submandibular gland (Menghi et al., 1984). Conversely, tubulo-acini of the posterior gland only contained mucous cells filled with secretory granules with a variable morphology, including bipartite structures characterized by an electron-lucent matrix and one or more electron-dense areas. Bipartite granules with two electron-dense spherules were found to be the most common; in addition, some granules showed an electron-dense cap similar to that of granules previously described in the lingual glands of chicken (Gargiulo et al., 1991).

As shown by conventional histochemical stainings for acidic groups, the rostral portion of the anterior lingual gland did not produce sialoglycoconjugates which, instead, were synthesized by the two other portions (Menghi et al., 1993). Lectin cytochemistry showed the occurrence of terminal α-D-GalNac which was heterogeneously distributed on the secretory granules of the anterior caudal and posterior lingual gland. Sialidase digestion showed the presence of terminal residues of sialic acids linked to α-D-GalNAc in the electron-lucent matrix of granules secreted by the posterior lingual gland. These findings suggest the occurrence of O-linked sialoglycoconjugates that appeared to be differently distributed in both the caudal portion of the anterior lingual gland and in the posterior gland. A small amount of terminal ß-D-Gal, linked to GalNAc, or GlcNAc was found both in electron-lucent and electron-dense secretory materials of the caudal anterior lingual gland. Sialic acid cleavage unmasked numerous sialoglycoconjugates with sub-terminal ß-D-Gal. In particular, in the posterior gland the presence of the terminal sequence sialic acid-ß-D-Gal and GlcNAc further supported the occurrence of N-linked sialoglycoconjugates.

Our ultrastructural studies showed that the secretory granules of quail lingual glands showed a biphasic nature, with a different glucidic composition in the

electron-dense and electron-lucent areas, also supported by lectin cytochemistry. As described by other authors for different systems (Castells et al., 1992, 1994; Leis et al., 1997) in quail lingual glands we also showed the presence of O-linked and N-linked oligosaccharides. The O-linked oligosaccharides are usually present on mucin-type glycoproteins of alimentary canal goblets cells (Slomiany et al., 1980), instead of N-linked oligosaccharides that are typical of all mucous cells. The concept of mucous and serous cells has been discussed by Schulte and Spicer (1983), who stated that not only the morphology, but also the cytochemical composition should be considered in classifying the secretory products of cells.

Actual findings further demonstrated that quail lingual glands produce sialoglycoconjugates characterized by heterogeneous composition also within granules of the same secretory cell. This heterogeneity of sialoglycoconjugates was also reported in the tongue of chicken anterior lingual glands (Menghi et al., 1992; Scocco et al., 1995). In addition, Samar et al. (2002) showed that the chicken palatine salivary glands secrete glycoproteins and sulphomucins in the lateral, and glycoproteins and carboxymucins in the medial, which are especially rich in sialic acid. Schulte and Spicer (1983) suggested that secretory cells may produce a mixture of different glycoproteins, or only one glycoprotein containing heterogeneous chains of oligosaccharides.

In conclusion, the cytological characteristics of quail lingual glands and the great variety of sialoglyco-conjugates that we have found, closely resemble what was previously established for chicken salivary glands. Differences with respect to mammalian salivary glands are likely ascribed to the diverse functional requirements in the physiology of the avian alimentary canal.

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