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Ultrastructure and Histochemical Study of the Lingual Salivary Glands of Some Bird Species

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Abstract.- Recent evidence supports the hypothesis that salivary glands develop independently of diet. However, histochemical features of the minor salivary glands appeared to be associated with the type of food ingested. We monitored ultrastructural and histochemical differences of lingual salivary glands among four species of birds, namely, *Egretta ibis, Gallus gallus, Buteo buteo* and *Anser anser*, by light and transmission electron microscopy. The respective diets of these species are very different. Periodic acid-Schiff reagent (PAS), alcian blue-PAS and bromo-phenol blue, and the carmine technique for neutral mucins were employed as the histochemical staining of the minor salivary glands of each species. The ultrastructure of secretory granules was heterogeneous and substructures were different among the four species. Histochemical analysis revealed the presence of sulfate groups and glycoconjugates, which appeared as the granules in these glands. These granules might represent of storage of secretory products that are located to the heterogeneous and complex ultrastructural patterns of granules in the mucous and seromucous cells.

Key Words: Birds, minor salivary gland, secretory granule, mucous and seromucous cells, glycoconjugates.

INTRODUCTION

Salivary glands, minor components of the lingual apparatus in birds have various chemical properties related to the feeding habits of the species. Relatively early investigations have already indicated that salivary glands vary markedly in terms of general morphology and histochemical composition (Nalavade and Varute, 1977; Pinkstaff, 1980; King and Mclelland, 1984; Suprasert *et al*., 1986). Nalavade and Varute (1977) performed histochemical studies of the nature and distribution of glycoconjugates secreted by the lingual glands of several species of birds, such as sparrow, kingfisher, parrot, spotted owl, pigeon and hawk. According to Pinkstaff (1980), the varying nature of the secretory cells, the presence of different cell types in the excretory duct system, and the organization of these components in the gland make it almost impossible to describe a typical salivary gland. Suprasert *et al.* (1986) investigated the secretions from mandibular and esophageal glands of chickens by light and electron microscopy. Recently, in various avian species, histochemical natures of secretory granules of the salivary glands have also investigated in combination with the locations and structures of the glands (Samar *et al.*, 1999; Jackowiak and Godynicki, 2005; Jackowiak and Ludwig, 2008; Guimarães *et al.*, 2009; Crole and Soley, 2010; Santos *et al*., 2011; Kadhim *et al.*, 2011; Erdoğan *et al.*, 2012; Sağsöz *et al*., 2013).

Studies by transmission electron microscopy (Tandler *et al.*, 1999; Jacob and Poddar, 1987; Kadhim *et al.*, 2011) demonstrated that secretory cells of salivary glands and other exocrine glands include granules of a variety of sizes and content. There may be focal deposits of dense material in a less dense matrix, randomly arranged filaments, bundles of parallel filaments, lamellae and microtubules, which lead to innumerable variations. Most studies of salivary glands by electron microscopy refer to mammals and reptiles but some descriptions of the structure and ultrastructure of the secretory granules in birds have been reported (Gargiulo *et al.*, 1993; Suprasert *et al.*, 1986; Samar *et al.*, 1999, Erdoğan *et al.*, 2012).

The goal of the present study was to describe different cells of the minor salivary glands of tongue of four species of birds, namely, *Egretta ibis, Gallus gallus, Buteo buteo* and *Anser anser*, with different

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diets to determine the qualitative effects of diet on secretory granules.

Table I.- Feeding and habitat of the four species of birds examined.

Class: Aves	Diet	Habitat
Family: *Ardeidae* *Egretta ibis* (cattle egret)	Carnivorous: Earthworms, worms and insects	Inhabits areas among feeding cattle in fields, marshes or in dry open country
Family: *Phasianidae* *Gallus gallus* (domestic fowl)	Vegetarian: grain, seeds and fruit and, sometimes, insects	Inhabits forests, woods, and dry scrub
Family: *Accipitridae* *Buteo buteo* (buzzard)	Carnivorous: rodents and small birds	Inhabits forests, woods, medium-altitude mountains, and wooded countryside.
Family: *Anatidae* *Anser anser* (graylag goose)	Herbivorous: plants, grains and aquatic plants	Inhabits marshlands, wetlands, estuaries, lakes, and dry cultivated fields.

MATERIALS AND METHODS

Animals

Four species of birds (five from each type) were used in the present study. Each species has a different respective ecological habitat and diet (Table I). All birds were obtained commercially, were mature, and in a good health. Birds were anaesthetized and decapitated according to the International Protocol for Biomedical Investigation with Human Being and Animals.

Histological staining

Sixteen tongues consisting of four from each species were removed immediately, and fixed in 10% neutral buffered formalin for three days. Then they were dehydrated in gradually increasing alcohol series, cleared in xylene, embedded in paraffin wax and cut at 7 □m serial sections. Some sections were stained with hematoxylin and eosin for general structure; other sections were stained with periodic acid-Schiff reagent (PAS), alcian blue-PAS and bromo-phenol blue, and the carmine technique for neutral mucins (Berger and Pizzolato, 1975), as shown in Table II.

Table II.- Histochemical staining and specificity.

Technique	Chemical groups identified
Periodic acid-Schiff (PAS)	Glycoconjugates with vicinal diol groups (glycoproteins and glycogen) (Pearse, 1968)
Carmine technique	neutral mucins (Berger and Pizzolato, 1975)
Alcian blue at pH 1.0	Sulfated glycoconjugates (Lev and Spicer, 1964)
Alcian blue at pH 2.5	Acidic glycoconjugates (Spicer *et al*., 1967)
Bromophenol blue	Total protein (Mazia *et al*., 1953)

Transmission electron microscopy

Four dissected tongues consisting of one from each species were fixed immediately in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 4 h. Then, they were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 2 h. They were then washed again in cacodylate buffer, dehydrated, and embedded in an epoxy-resin araldite mixture. Semi-thin sections were cut by ultramicrotome and stained with toluidine blue and then examined. Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (CX100; JEOL, Tokyo) at an accelerating voltage of 60 kV.

RESULTS

Histology

The paired gland, lingual glands of *E. ibis*, was attached to the dorso-lateral surface of the posterior half of the paraglossum. Septa divided the gland into lobules (Fig.1A). In *G. gallus*, the anterior lingual glands were located in the dorso-

lateral angles of the tongue. The posterior lingual glands were identified as the paired structure located at the base of the tongue (Fig. 1B). The free portion of the tongue in *A. anser* contained the anterior and posterior lingual glands. The former were situated on the lateral surface of the tongue and the latter were found at the base of the tongue, extending to the larynx (Fig. 1C). The lingual gland in *B. buteo* was a paired elongated gland in the posterior end of the tongue (Fig. 1D).

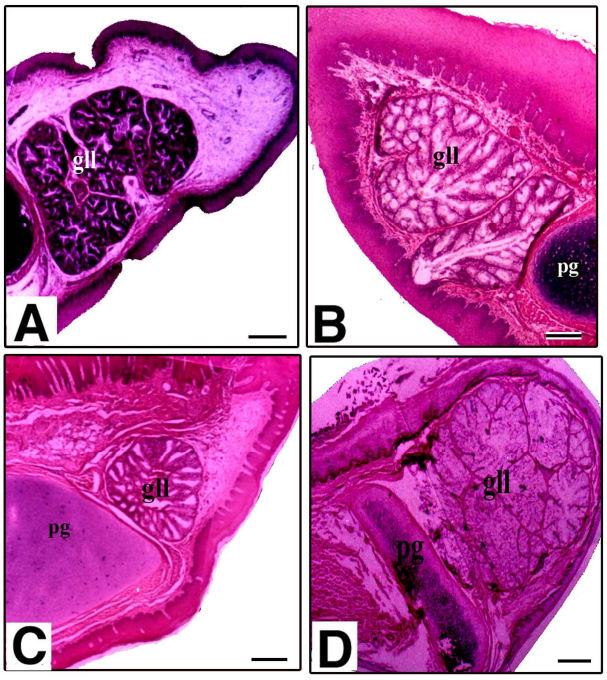

Fig. 1. Histological structure of the tongue of four avian species. A, transverse section through the posterior free portion of the tongue of *E. ibis* showing lingual glands (gll). B, transverse section through the posterior region of the free portion of the tongue of *G. gallus* showing lingual glands (gll) and paraglossale (pg). C, transverse section through the posterior region of the free portion of the tongue of *A. anser* showing lingual glands (gll) and paraglossale (pg). D, transverse section through the posterior region of the free portion of the tongue of *B. buteo* showing lingual glands (gll) and paraglossale (pg). All scale bar = 10 µm, H & E staining.

Ultrastructural features

In *E. ibis*, the granules of the mucous cells in the lingual glands were homogeneous, with varying electron density. The electron-lucent granules had regions low and high density (Fig. 2A). In *G. gallus*, the glands consisted of secretory epithelium with seromucous cells in the anterior lingual zone while the cells in the posterior region contained only mucous and pale granules that were larger than those in the seromucous cells. Although most of the granules were electron-lucent, there were variations in electron density (Figs. 2B,C). In *A. anser*, the anterior glandula lingualis contained mucosecretory cells with granules of moderate electron density (Fig.2D). The posterior lingual glands included cells filled with fine granules (Fig.2E). In *B. buteo*, the secretory cells contained large numbers of irregular undulating granules. The matrix of these granules included deposits of moderate density (Fig. 2F).

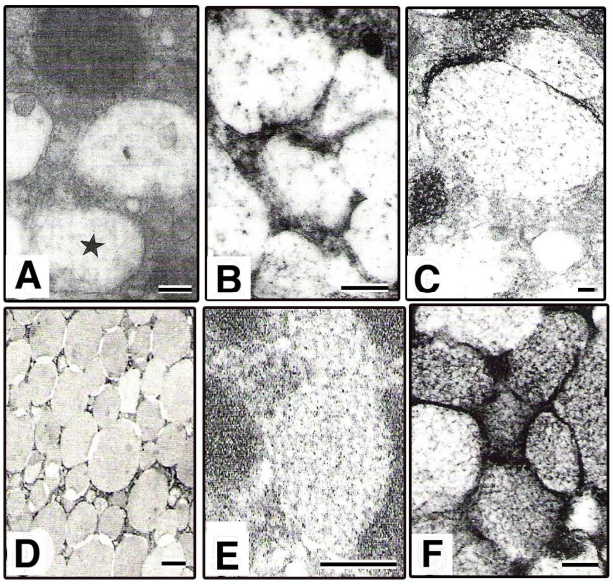

Fig. 2. Transmission electron micrographs of the lingual gland of four avian species. A, the posterior lingual gland of *E. ibis* showing granules in mucous cells (asterisk) with varying degrees of electron density. B, the anterior lingual gland of *G. gallus* showing seromucous cells. C, the posterior lingual gland of *G. gallus* showing mucous cells. D, the anterior lingual gland of *A. anser* showing mucous cells. E, the posterior lingual gland of *A. anser* showing mucous cells. F, the posterior lingual gland of *B. buteo* showing large irregular granules and electron dense substructures. All scale bar = 1µm.

Histochemistry

The lingual gland of *A. anser* stained more strongly with PAS than those of the other three species examined (Fig. 3A). The lingual gland was most intensely stained with alcian blue in *G. gallus* (Fig. 3B), strongly stained in *A. anser*, and moderately stained in *E. ibis* and *B. buteo*. Bromo-phenol blue staining yields a strong positive reaction in the secretory granules of *E. ibis* and *B. buteo* (Fig. 3C) but only moderate or weakly positive reaction in *G. gallus* and *A. anser* respectively. The intensity of carmine staining, which is specific for neutral mucin, was very strong in the lingual gland of *G. gallus*, strong in *E. ibis* (Fig. 3D) and *B. buteo*, and moderate in *A. anser*. The results of histochemical staining are summarized in Table III.

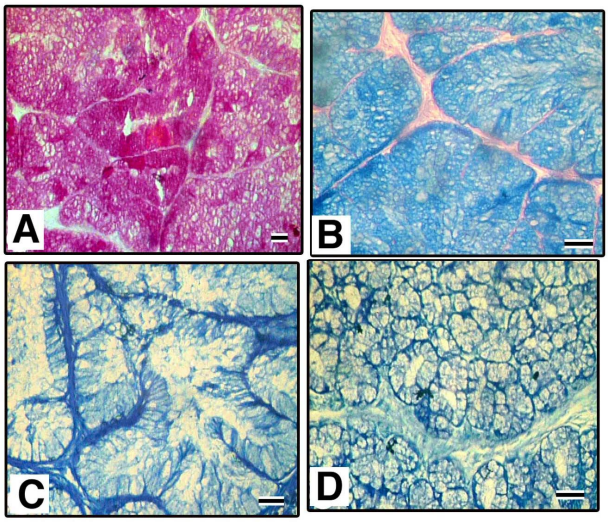

Fig. 3. Histochemical micrographs of the lingual gland of four avian species. A, PAS staining of the posterior lingual gland of *A. anser*. B, Alcian blue staining of the anterior lingual gland of *G. gallus*. C, Bromo-phenol blue staining of the posterior lingual gland of *B. buteo*. D, Carmine staining of the posterior lingual gland of *E. ibis*. All scale bar = 20 µm.

DISCUSSION

We analyzed the histology, histochemistry and ultrastructure of the minor salivary glands of four species of birds with different feeding habits, because the minor salivary glands have been often observed around the mouth in various species of birds. Minor salivary glands, which secrete abundant mucins, are present in the newborn human, chick , quail and emu (Samar *et al.*, 1991, 1993; Capacchietti *et al*., 2009; Crole and Soley, 2010), suggesting a genotypic evolution independent of glandular adaptation to the type of diet, in contrast to earlier suggestions (Farner and Ziswiller, 1972; Crole and Soley, 2009, 2010). Development of the salivary glands in avian species has been related, classically, to the type of diet. Early reports noted that granivorous birds fed on dry food had larger salivary glands than those of rapacious species (Farner and Ziswiller, 1972). However, also in piscivorous birds, whose food is naturally lubricated, the minor salivary glands were described as relatively developed organs (Samar *et al.*, 1995, 1999). Our analysis revealed that the present avian species (*E. ibis*, *G. gallus*, *B. buteo* and *A. anser*) have well-developed lingual salivary glands, in accordance with the results of the palatine, linguolaryngeal and posterior lingual glands of the Magellanic penguin (Samar *et al.*, 1999), the alveolar gland of the kelp gull (Samar *et al.*, 1995), the palate gland of the chicken (Samar *et al.*, 2002) , of the lingual and palate glands of the chukar partridge (Erdoğan *et al*., 2012; Sağsöz *et al*., 2013), the lingual gland of the emu (Crole and Soley, 2009) , the ostrich (Jackowiak and Ludwig, 2008; Guimarães *et al*., 2009) and the white tailed eagle (Jackowiak and Godynicki, 2005) and of the lingual glands of large part of the lingual mucosa of the rhea (Santos *et al*., 2011). However, our results do not agree with those of Farner and Ziswiller (1972), who reported that the salivary glands of fish-eating birds are poorly developed. The discrepancy between results might be due to genetic variations in the different avian species, but further research is

Table III.- Reactivity to histochemical staining.

Species	PAS	Alcian blue	Bromo-phenol blue	Carmine technique
E. ibis	++	++	++++	+++
G. gallus	+++	++++	++	++++
A. anser	++++	+++	+	++
B. buteo	+	++	++++	+++

++++, sever reaction; +++, strong reaction; ++, moderate reaction; +, weak reaction.

needed for fuller clarification.

We found glycoconjugates that contained vicinal diol and sulfate groups, neutral mucins and acidic glycoconjugates in the secretory granules of the four species that we examined. The strong staining with PAS of the lingual glands of *G. gallus* and *A. anser* reveal vicinol diols in mucous fluids composed of different types of mucins, as reported in the lingual glands of the chicken (Gargiulo *et al.*, 1991; Kadhim *et al.*, 2011), *Larus ridibundus* (Zaccone, 1977), kingfisher, parrot, sparrow, pigeon (Nalavade and Varute, 1977), quail (*Coturnix coturnix japonica*) (Menghi *et al*., 1993) and collared dove (*Streptopelia decaocta*) (Taib and Jarrar, 2001). The staining of secretory granules in mucous cells with a periodic acid-thiocarbohydrazide-silver proteinate sequence indicated the presence of glycoconjugates with sulfate, carboxylate and vicinal diol groups and of more complex glycoconjugates that were preferentially located in the core of the granules (Gargiulo *et al.*, 1991; Taib and Jarrar, 2001; Kadhim *et al.*, 2011). The presence of neutral and acidic mucopolysaccharides in the salivary glands of the chicken was suggested by Rangel *et al*. (1972). More recently, Suprasert *et al.* (1986) noted the presence of glycoconjugates that contained sulfate, carboxylate and vicinal diol groups in the mandibular glands of chicken.

We observed moderate and weak staining with PAS in lingual glands of *E. ibis* and *B. buteo*, respectively, in contrast to the results reported by Al-Mansour and Jarrar (2007) in the little egret *(Egretta garzetta)*, in which no PAS staining was observed and in which lingual glands appeared to be devoid of glycogen and neutral mucosubstances. PAS-positive reaction in the secretory units and ducts of the lingual glands in the partridge (*Alectoris chukar*) demonstrates the presence of neutral, sialidase-labile sialomucins and weakly sulfated acidic mucins (Erdoğan *et al.*, 2012). The lingual glands were most intensely stained with alcian blue in *G. gallus*, in the present study. They were strongly stained in *A. anser* and moderately stained in *E. ibis* and *B. buteo*, as reported also in the little egret *(Egretta garzetta)*. These glands appeared to secrete acid mucosubstances (Taib and Jarrar, 2001; Al-Mansour and Jarrar, 2007). The lingual glands of the bulbul and emu were reported to be have a mixture of serous- and mucous-type salivary glands (Al-Mansour and Jarrar, 2004; Crole and Soley, 2009, 2010). In the partridge (*Alectoris chukar*) (Erdoğan *et al.*, 2012), the alcian blue reaction was more intense in the anterior lingual glands than in the posterior lingual glands.

In the present study, the lingual glands reacted most intensely to carmine staining in *G. gallus*, strongly in *E. ibis* and *B. buteo* and moderately in *A. anser*. PAS-diastase and Best carmine staining revealed that, unlike those in the chicken (Arthitvong *et al.*, 1999) but similar to those in the bulbul (*Pycnonotus leucogenys*) (Al-Mansour and Jarrar, 2004), neither the anterior nor the posterior lingual glands of the partridge (*Alectoris chukar*) contain glycogen (Erdoğan *et al.*, 2012).

Our ultrastructural analyses revealed that secretory granules varied in composition, with differences in substructure among the four species examined. Heterogeneous ultrastructure of secretory granules in salivary glands has been described in avian species. The structure of granules is related to the progress of the production, storage and secretion of the granules and this pathway is influenced by diet (Phillips *et al.*, 1993; Tandler and Phillips, 1998; Crole and Soley, 2009, 2010). Ultrastructural differences in the granular structure of the parotid glands were reported between mice that had been fed and mice that had been starved for 12 hours (Pinkstaff, 1980). The different types of mucin that are found during the maturation process correspond to the varying electron densities of granules in the human lingual gland (Pinkstaff, 1980; Olmedo *et al.*, 2000).

As suggested by the present and previous results (Samar *et al.*, 1991, 1993, 1999; Al-Mansour and Jarrar, 2007; Kadhim *et al.*, 2011; Erdoğan *et al.*, 2012), it seems highly probable that salivary glands contribute to maintenance of the oral environment (Crole and Soley, 2009, 2010; Kadhim *et al.*, 2011), with interactions with soft tissues via the chemical components of their mucins. These interactions include (1) lubrication and the moistening of food (Liman *et al*., 2001; Kadhim *et al.*, 2011); (2) maintenance of the integrity of the buccal mucus via the properties of mucins (low solubility, high viscosity, elasticity, and adhesion),

which form a protective pellicle against desiccation and external physical factors (Liman *et al*., 2001; Samar *et al*., 2002); and (3) difference by mucins with a high degree of sulfation of the development of buccal pathogenic flora. Among the constituents of salivary gland secretions, mucins play a dominant role in maintaining an appropriate oral microenvironment, interacting with hard and soft oral tissues and with bacterial flora (Samar *et al*., 1993; Tabak, 1995, Schimming and Vicentini, 2000).

We can conclude from the present study that the ultrastructural and histochemical differences in lingual salivary glands among the four avian species examined might represent specific mode of storage of secretory products that determine the heterogeneous and complex ultrastructural features of the granules in their respective mucous and seromucous cells.

Further investigations of avian salivary glands might reveal whether the structures and secretions of these glands are related to the phylogeny and/or the feeding habit.

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