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Confocal evaluation of native and induced lectin binding contributes to discriminate between lingual gland glycocomponents in quail

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Summary. A confocal analysis was performed on the quail (*Coturnix coturnix japonica*) lingual salivary glands where the carbohydrate chains were studied by lectin histochemistry. For this purpose, appropriate FITC- and TRITC-conjugates were used for double binding also accomplished with sialidase digestion. The glycosidic components of the quail lingual salivary glands were found to be heterogeneously distributed on the different secretory structures as well as on the single secretory elements of each adenomere. The rostral portion of the anterior lingual gland was found to only secrete neutral glycocomponents, characterized by terminal ß-galactose, N-acetylgalactosamine and fucose residues in contrast to the caudal portion that was shown to be extremely heterogeneous and to produce sialylated glycoconjugates characterized by the terminal sequences sialic acid-ß-galactose-N-acetylgalactosamine, sialic acid-ß-galactose-N-acetylglucosamine, and sialic acid-α-N-acetylgalactosamine partly codistributed within secretory adenomeres. The posterior lingual gland was observed to be the major contributor to the secretion of salivary mucins containing sialoglycoconjugates with terminal sialic acid residues linked to ß-galactose-N-acetylgalactosamine or α-N-acetylgalactosamine often located in distinct secretory elements.

Key words: Carbohydrates, Lectins, Confocal microscopy, Quail, Lingual glands

Introduction

Glycoconjugates are a heterogeneous class of macromolecules whose properties are strongly influenced by disposition, number and type of glucidic residues exposed on their surface (Montreuil, 1980). A variety of glycoproteins have been demonstrated in the mammalian major salivary glands. Mucous secretions contain O-glycosylated as well as N-glycosylated glycoproteins of high and low molecular weight. The monosaccharides present in the glycans main chain are N-acetylgalactosamine, N-acetylglucosamine, glucose, mannose, L-fucose, D-galactose, and sialic acid. The incorporation of sugar moieties into glycoproteins involves the sequential action of genetically determined sugar transferases; therefore, the differences in glycoprotein synthesis by different salivary gland cells may result in heterogeneity of the carbohydrate content (Tabak, 1995).

Lectins are sugar-binding proteins or glycoproteins of non-immune origin, which agglutinate cells and/or precipitate glycoconjugates having saccharides with appropriate complementary sequences; accordingly, the affinity of different lectins to specific sugars make them useful as histochemical probes (Schulte and Spicer, 1983; Schulte, 1987; Spicer and Schulte, 1988; Menghi et al., 1989, 1999; Menghi and Materazzi, 1994; Garrett et al., 1996; Hirshberg et al., 1996).

Avian salivary glands produce a great quantity of glycoconjugates (Nalavade and Varute, 1977; Zaccone, 1977; Suprasert et al., 1986; Gargiulo et al., 1991; Gheri Bryk et al., 1992; Menghi et al., 1992) but, compared to the corresponding macromolecules of mammals, show differences about sugars in the core region of the carbohydrate chains (Scocco et al., 1995).

The present paper is an extension of previous histochemical studies on quail salivary glands (Menghi et al., 1993) and aims to survey the location of the carbohydrate chains and to evaluate the distribution patterns as well as the spatial disposition of glycoconjugates in the anterior and posterior lingual glands. For this purpose, we performed lectin histochemistry by single and double labeling, also combined with sialidase digestion and then analyzed the lectin receptor sites at Confocal Laser Scanning Microscope (CLSM) level.

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Materials and methods

Tissue collection and processing

Ten female quail (*Coturnix coturnix japonica*), two months old, were killed according to the recommendation of the Italian Ethical Committee. Immediately after removal, small pieces of tongues were fixed, for 3h at 4 °C, in a solution of 4% paraformaldeyde, 1% glutaraldeyde and 0.2% picric acid in 0.1M phosphate buffer, pH 7.6, containing 0.5mM $CaCl_2$. Specimens were then rinsed with phosphate buffer with 3.5% sucrose added, postfixed, after quenching of free aldehydes, for 2 h at 4 °C in 2% uranyl acetate in 0.1M maleate buffer, pH 6.0, containing 3.5% sucrose, dehydrated with acetone up to 90% and finally embedded in Unicryl resin (Scala et al., 1992) under the condition previously detailed (Menghi et al., 1996). Sections (3 μm thick) were collected on superfrost glass slides opportunely cleaned.

Lectin labeling

Specimens were treated for 30 min with 0.05M PBS, pH 7.4, with 0.03% Triton X-100 and 1% BSA added. After three rinsings, 5 min each, in PBS, they were incubated for 90 min at room temperature with FITC or TRITC labeled lectins opportunely combined and diluted in PBS-Triton-BSA. Samples were finally washed three times, 10 min each, and mounted in a mixture of PBS/glycerol (1:1) on Top-watermount. Single and double, sequential or simultaneous, labeling methods were performed using the following selected lectins (Sigma): PNA (from *Arachis hypogaea*, ß-D-Gal-[1-3]-D-GalNAc) diluted 1:50; DBA (from *Dolichos biflorus*, α-D-GalNAc) and PSA (from *Pisum sativum*, α-Man), diluted 1:20; WGA (from *Triticum vulgaris*, ß-GlcNAc>>NeuNAc); ECA (from *Erythrina cristagalli*, ß-D-Gal-[1-4]-D-GlcNAc) and Con A (from *Canavalia ensiformis*, α-Man>α-Glc) diluted 1:40; LTA (from *Lotus tetragonolobus*, α-L-Fuc) and UEA I (from *Ulex europaeus*, α-L-Fuc) diluted 1:10.

Image analysis

Sections were observed on a Biorad Ar/Kr Bio-Rad MRC-600 Confocal Laser Scanning Microscope (Bio-Rad, Hertfordshire, U.K.) connected to a Nikon Diaphot-TMD-EF inverted microscope equipped with a Plan Apo oil-immersion objective (x10; NA=1.4). The image acquisition was carried out by Bio-Rad COMOS software. Microphotographs were taken using a Polaroid Quick Print VI-350 Video Printer equipped with a 35 mm camera (Velvia Fujichrome film).

Sialidase digestion

This was performed by treating sections for 6 h at 37 °C with a solution of 0.5 U/ml of the enzyme (neuraminidase type V, Sigma, from *Clostridium perfringens*) in 0.1M sodium acetate buffer, pH 5.5, containing 10mM $CaCl_2$ (Menghi et al., 1989).

Controls

Control experiments were carried out by incubating lectins with the corresponding inhibitory sugars (0.2-0.4M) and testing unconjugated lectins.

Controls for sialidase digestion were carried out by treating adjacent sections for 6 h at 37 °C with sialidase-free buffer.

Results

Anterior lingual gland. Rostral portion

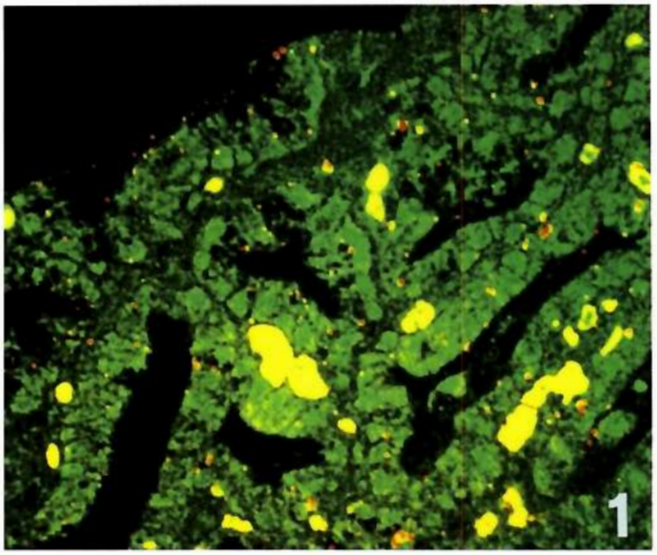
The secretory cells of the rostral portion showed modest affinity for PNA-TRITC and ECA-FITC lectins (Fig. 1), DBA-FITC, and WGA-TRITC. Sialic acid cleavage did not change the binding intensity of these lectins. Con A-FITC and PSA-TRITC lectins exhibited codistribution of binding sites at cytoplasm level of all cells (Fig. 2); LTA-TRITC and UEA I-FITC lectins also stained numerous cells and the reactive sites were codistributed (Fig. 3).

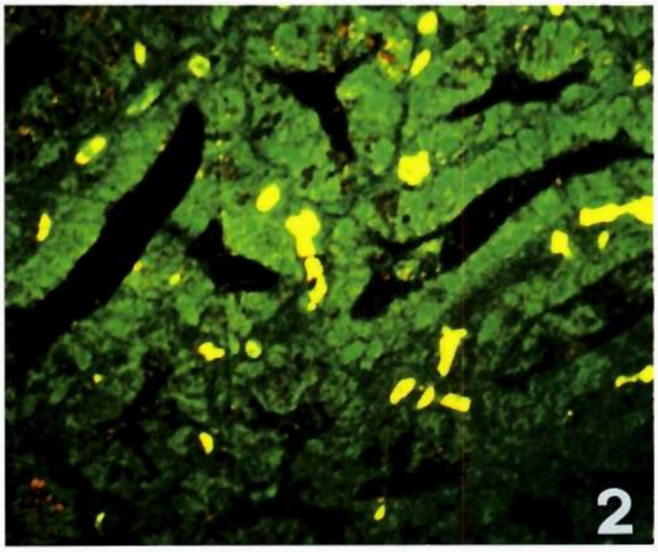
Anterior lingual gland. Caudal portion

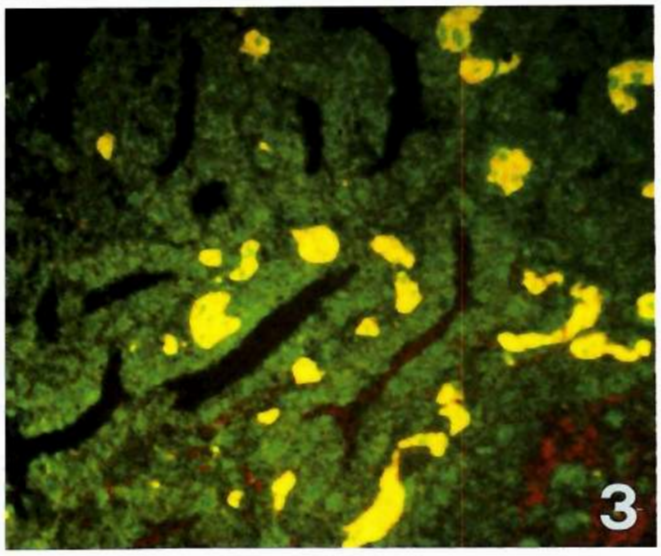
DBA-FITC showed some heterogeneously-distributed positive cells (Fig. 4); sialidase digestion strongly enhanced the DBA-FITC affinity on the great part of the secretory cells (Fig. 5a). Conversely, this region did not show PNA-TRITC and ECA-FITC affinity sites in the secretory elements (Figs. 4, 6). Sialidase digestion induced numerous PNA-TRITC reactive sites (Fig. 7b) clearly evidenced by examining the merged images (Fig. 5b, 7c) that gave additional information about the differential location as well as the co-presence of penultimate ß-galactose residues linked to different internal acceptor sugars. Sialic acid removal also promoted the ECA-FITC binding (Fig. 7a) that in some cells appeared to be colocalized with sialidase/PNA reactive sites (Fig. 7c). Con A-FITC, WGA-TRITC and PSA-TRITC only faintly stained this glandular portion. LTA-TRITC and UEA I-FITC did not react at this level, except for intercellular boundaries.

Posterior lingual gland

ECA-FITC lectin did not stain secretory cells of the posterior lingual gland (Fig. 8) and sialidase digestion did not increase the lectin binding. Scarce PNA-TRITC positive sites were located at seromucous cells (Fig. 8) that, instead, became strongly and heterogeneously stained after sialidase pretreatment (Fig. 11b). A few DBA-FITC reactive cells were located on this portion







(Fig. 9a) but sialic acid removal induced an increased affinity in most cells (Fig. 11a). WGA-TRITC binding sites were found at this level with different intensity within secretory adenomeres (Fig. 9b). Con A-FITC and PSA-TRITC lectins only faintly stained the secretory cells. UEA I-FITC and LTA-TRITC lectins were only located at the intercellular boundaries of the seromucous tubules (Fig. 10).

Discussion

Single and double lectin labeling, combined with sialidase digestion and analyzed at CLSM level, allowed us to visualize terminal sugars, specific carbohydrate sequences and distribution of different residues in the glandular structures of the quail tongue. Actual results substantially confirmed findings originated from the application of horseradish peroxidase conjugated lectins (Menghi et al., 1993) and gave additional information about the spatial arrangement of secretory glycoconjugates.

In particular, it was found that the rostral portion of the anterior lingual gland is composed of a homogeneous cell population, while the caudal portion as well as the posterior lingual gland are formed by heterogeneous cell populations characterized by different sialoglycoconjugates.

As shown by conventional histochemical stainings for acid groups (Menghi et al., 1993), the rostral portion of the anterior lingual gland did not produce sialoglycoconjugates that, instead, were abundantly synthesized by the two other portions and were found to be very heterogeneous with respect to the penultimate and the last but two sugars. N-linked fucoglycoconjugates, rich in mannose and devoid of sialic acid, are copious in the rostral region of the anterior lingual gland where a codistribution with PSA, indicating the occurrence of a fucosylated core region, was also observed.

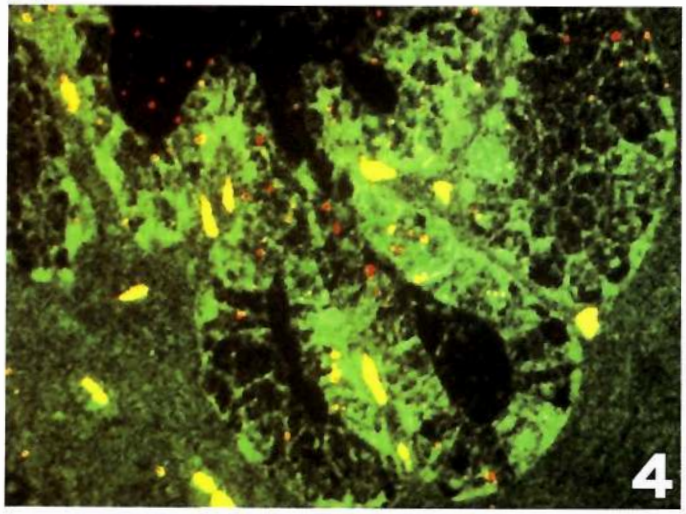
The comparison of PNA and ECA binding patterns showed that the terminal ß-galactose can be linked to N-acetylgalactosamine with 1-3 glycosidic bond or to N-acetylglucosamine with 1-4 glycosidic bond and that glycoconjugates containing terminal galactose are only produced by the secretory elements of the posterior gland.

Sialic acid cleavage unmasked a lot of ß-galactose

Fig. 1. Anterior lingual gland. Rostral portion. Double labeling with PNA-TRITC and ECA-FITC. Colocalization of various sites is present in tubular cells. x 850

Fig. 2. Anterior lingual gland. Rostral portion. Double labeling with Con A-FITC and PSA-TRITC. The merged image supports a colocalization of receptor sites reflecting a homogeneous cell population. x 850

Fig. 3. Anterior lingual gland. Rostral portion. Double staining with LTA-TRITC and UEA I-FITC lectins that show colocalization within secretory cells. x 850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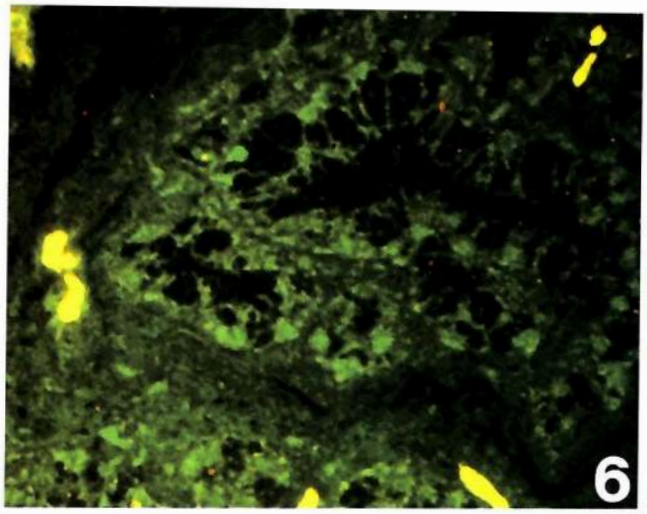
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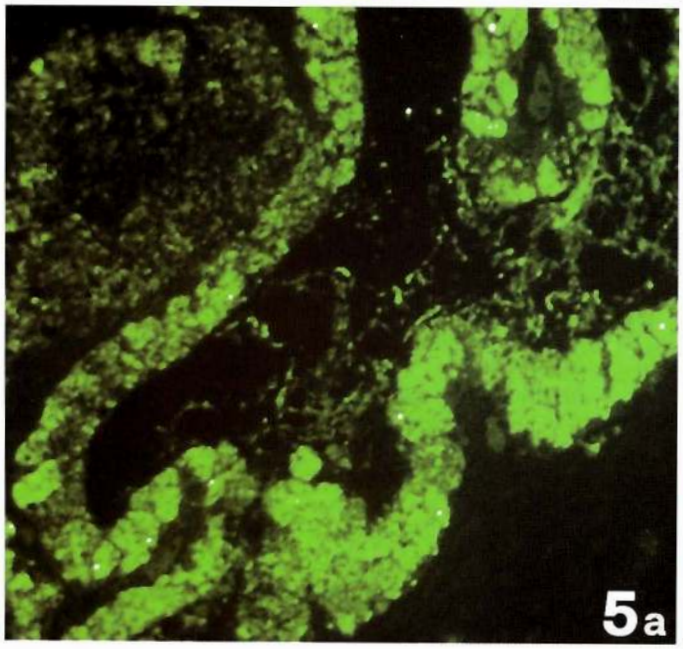
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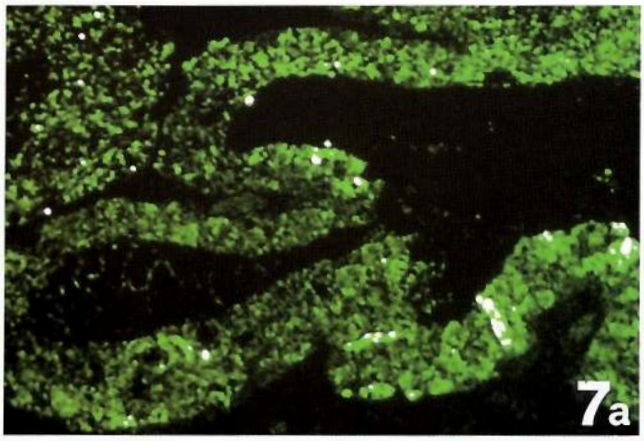
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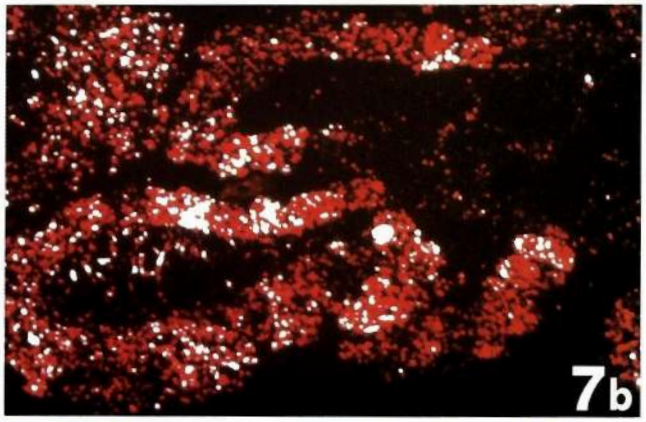
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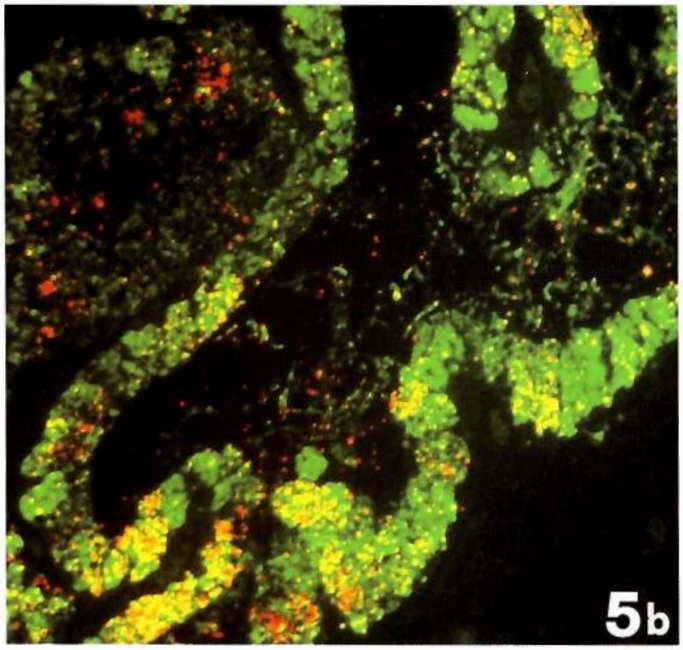
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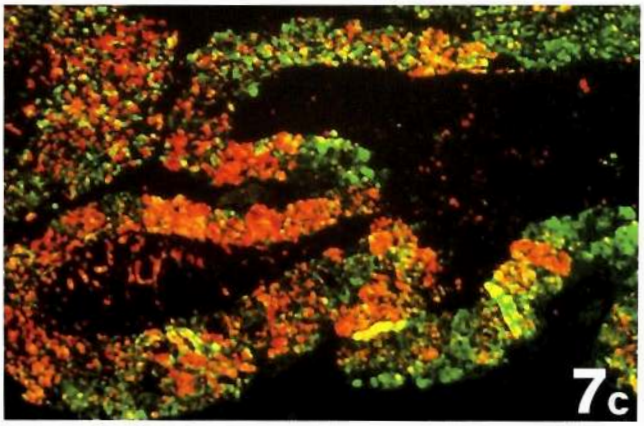
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Fig. 4. Anterior lingual gland. Caudal portion. Double staining with PNA-TRITC and DBA-FITC. The merging of images clearly illustrates the only presence of a few DBA-stained cells (green). x 850

Fig. 5. Anterior lingual gland. Caudal portion. Double staining. **a.** Sialidase DBA-FITC. Note the appearance of numerous DBA-reactive sites (green) that interest almost all the tuboalveolar secretory cells. The sialidase PNA-TRITC staining (red) is not shown. **b.** The processing of sialidase DBA-FITC + PNA-TRITC images to give merges evidences the occurrence of a modest cell population (yellow staining) secreting both the terminal disaccharides sialic acid-α-N-acetylgalactosamine and sialic acid-ß-galactose. x 850

Fig. 6. Anterior lingual gland. Caudal portion. Double staining with PNA-TRITC and ECA-FITC reveals the lack of terminal ß-galactose residues in the secretory cell cytoplasm. x 850

Fig. 7. Anterior gland. Caudal portion. Double staining. **a.** Sialidase ECA-FITC. **b.** Sialidase PNA-TRITC. **c.** Sialidase PNA-TRITC + ECA-FITC. After removal of terminal sialic acid, penultimate galactose-1,4-N-acetylglucosamine (green) and penultimate galactose-1,3-N-acetylgalactosamine (red) residues become accessible to ECA and PNA lectins, respectively. The merging of images reveals the occurrence of differentially localized (red or green) and colocalized (yellow) sites in the secretory adenomeres. x 850

and N-acetylgalactosamine residues, recognized by the respective specific lectins, in both the caudal portion of the anterior lingual gland and the posterior lingual gland. The co-presence of the terminal sequence sialic acid-ß-galactose and the overlapped WGA binding sites strongly support the occurrence of N-linked sialoglycoconjugates. In addition, the presence of the terminal sequence sialic acid-α-N-acetylgalactosamine indicates the occurrence of O-linked sialoglycoconjugates that appeared to be differentially distributed in both the caudal portion of the anterior lingual gland and in the posterior gland. After sialidase digestion, PNA and ECA receptor sites evidenced, in the caudal portion, numerous cells producing heterogeneous sialoglycoconjugates with sub-terminal ß-galactose linked to N-acetylgalactosamine (sialidase/PNA) and N-acetylglucosamine (sialidase/ECA); conversely, sialoglycoconjugates, characterized by the sub-terminal N-acetylgalactosamine, were found to be uniformly distributed as indicated by the sialidase/DBA-induced staining. Heterogeneous cell population involved in the synthesis of sialoglycoconjugates with sialic acids subtended to ß-galactose or N-acetylgalactosamine were found in the posterior gland.

This heterogeneity in sialoglycoconjugates can be related to the specific activity of sialyl transferases that, probably in relation to the functional moment, can produce some sialylated sequences rather than others and influence the mucin composition and properties (Paulson and Colley, 1989; Accili et al., 1994, 1996).

Differences in both morphology and carbohydrate histochemistry of the quail salivary glands could reflect the heterogeneity of mammal salivary glands, as shown by the presence of three glands which differ in structure and secretion type. The rostral portion is similar, for the secretory product composition, to the parotid gland that produces less viscous saliva; the other seromucous and mucous glands are comparable to the submandibular and sublingual glands.

Fucoglycoconjugates responsible for the mucin hydrophobia seem not to be represented in the mucous structures; accordingly, they are present, in modest amount, in the tubules of the rostral portion of the anterior lingual gland where they could counterbalance the fluidity of secretory products. Finally, the modest occurrence of secretory fucose residues, the most hydrophobic carbohydrate, and the relevant amount of sialylated chains in the mucous secretory cells may be related to the necessity of fluid saliva production in birds.

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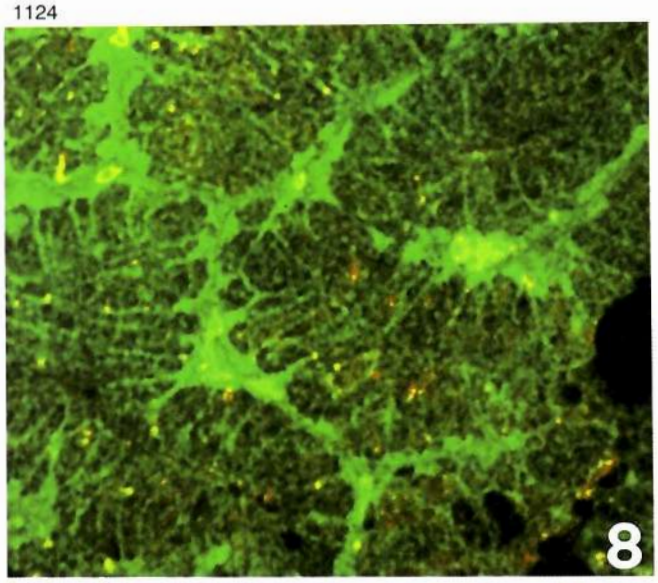
Fig. 8. Posterior lingual gland. Double staining with PNA-TRITC and ECA-FITC shows the presence of a few terminal galactose linked to N-acetylgalactosamine (red) in the secretory cells and the occurrence of terminal galactose linked to N-acetylglucosamine (green) in the intercellular boundaries and connective tissue. x 850

Fig. 9. Posterior lingual gland. Double staining with DBA-FITC and WGA- TRITC. **a.** Terminal α-N-acetylgalactosamine residues are present in a few secretory cells. **b.** The merged image evidences that almost all the mucous cells contain N-acetylglucosamine residues. x 850

Fig. 10. Posterior lingual gland. Double staining with UEA I-FITC and LTA-TRITC indicates the lack of terminal fucose residues in the mucous cells. x 850

Fig. 11. Posterior lingual gland. Double staining. **a.** DBA-FITC staining after sialidase digestion reveals the presence of heterogeneously distributed preterminal α–N-acetylgalactosamine residues. **b.** Sialidase PNA-TRITC + DBA-FITC. Note the mosaic lectin patterns reflecting the differential secretion of sialoglycoconjugates characterized by the terminal disaccharides sialic acid N-acetylgalactosamine (green) and sialic acid galactose-1,3-N-acetylgalactosamine (red). x 850



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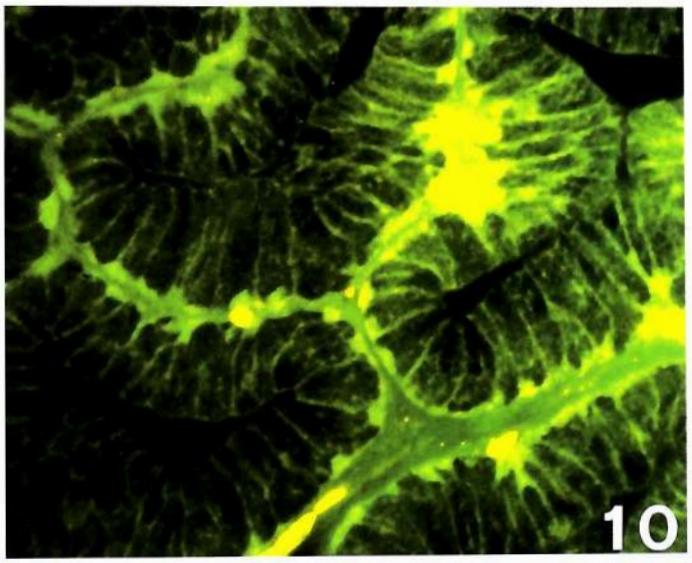

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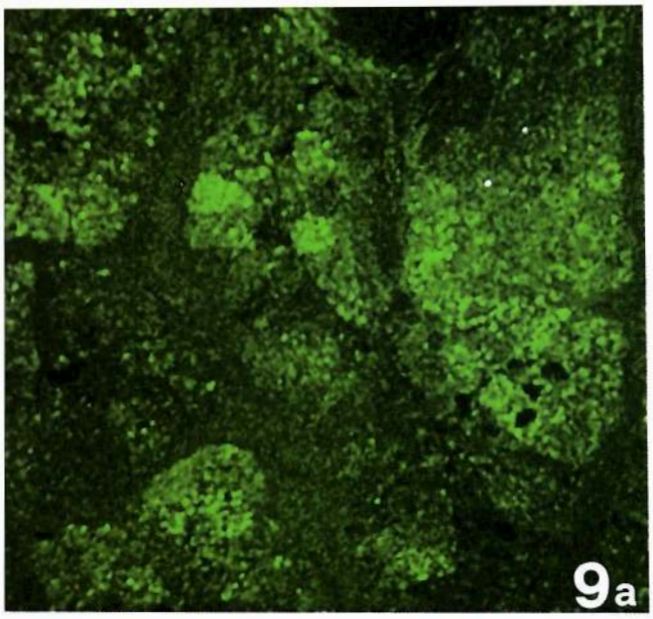

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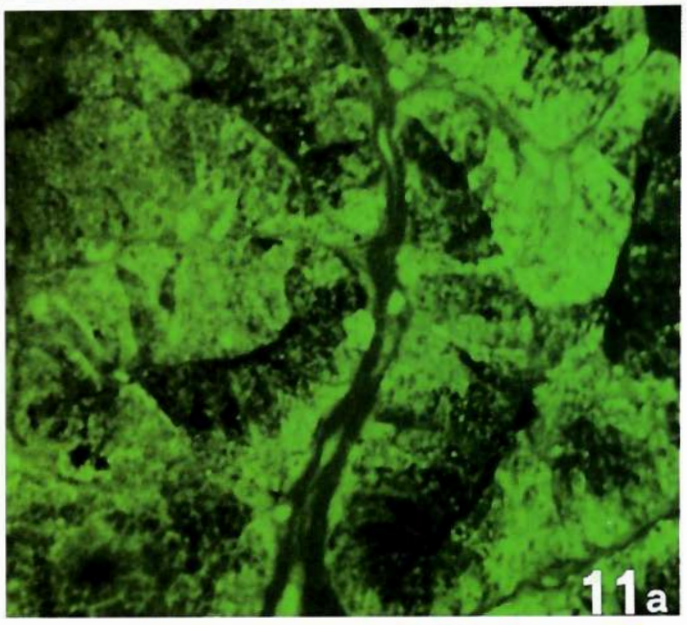

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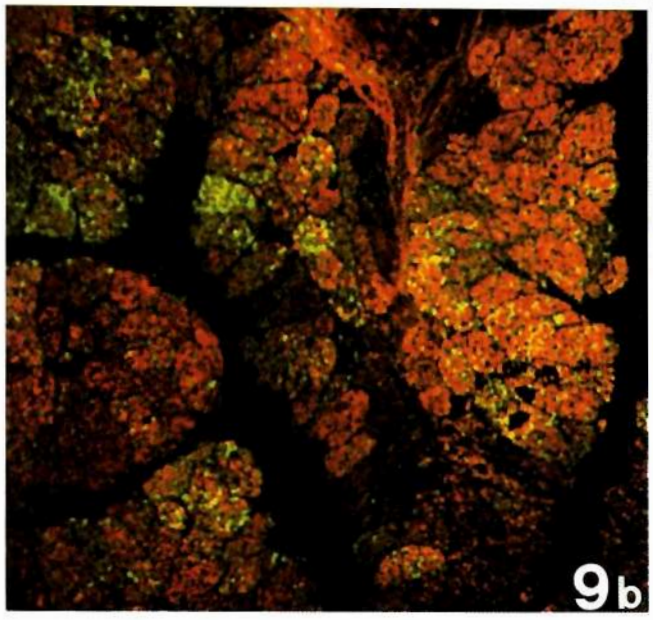

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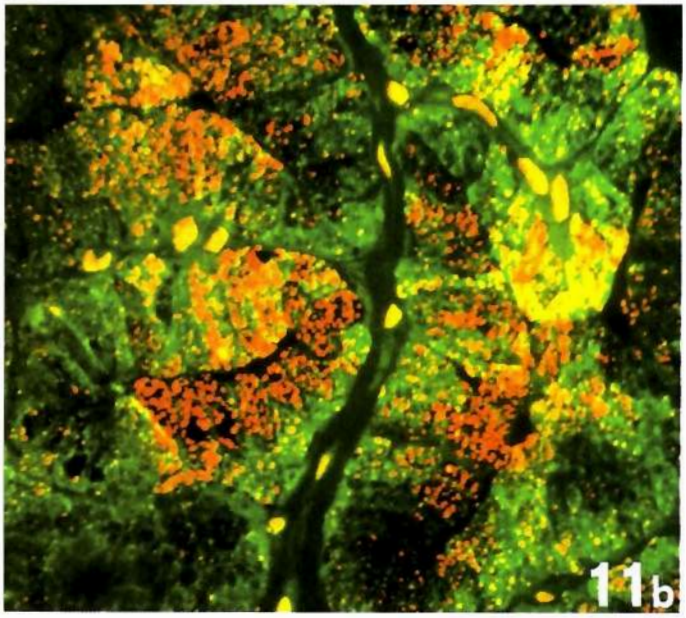

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