Histol Histopath (1994) 9: 723-731

Histology and Histopathology

Variety of sialic acids occurring in the bovine sublingual gland

D. Accili, M.G. Gabrielli and G. Menghi
Dipartimento di Biologia M.C.A., Università di Camerino, Camerino, Italy

Summary. Sialoglycoconjugates were investigated in the bovine sublingual gland by direct visualization of sialic acid with specific lectins (LPA, SNA) and by histochemical procedures combined with sialidase digestion and lectins. The most reactive histological sructures were found to be acini which contained glycoconjugates with terminal disaccharides consisting of sialic acid linked to galactose or N-acetylgalactosamine. Resistance to periodate oxidation was interpreted as demonstrating a relevant presence of C_7, C_8 and C_9 acetylated sialic acids. KOH-Sialidase-DBA and KOH-Alcian blue sequences allowed the identification of C_4 acetylated sialic acids.

Key words: Bovine, Sublingual gland, Sialic acids, Lectins, Sialidase

Introduction

Sialic acids are a group of 9-carbon sugars usually found in several kinds, varying in degree, of O-acetylation and N-glycolylation. As terminal residues sialic acids represent the common sugars of glycoproteins and glycolipids where they seem to determine the stop for lengthening the oligosaccharide chains (Pigman, 1977).

It has been hypothesized that the great variety of sialic acids is correlated to the different properties which these components have in biological matrices (Montreuil, 1980; Reutter et al., 1982; Schauer, 1982; Schulte and Spicer, 1985; Schulte et al., 1985). The knowledge of the types of sialic acids and the linkage of sialic acid to the subterminal sugars is the key to elucidate the function of sialoglycoconjugates. Accordingly, interest in O-acetylated sialic acids is increasing, since they have been recognized to play a role in a variety of physiological and pathological processes (Schauer, 1987; Devine et al., 1991; Mancini et al., 1991; Werner et al., 1991; Dall'Olio et al., 1992).

In particular, salivary glycoproteins have been described as an important source of sialic acids which show a heterogeneous structure. For example, the porcine submandibular gland is rich in N-glycolylneuraminic acid, whereas ovine and bovine submandibular glands present prominent N-acetylneuraminic acid (Pigman, 1977; Corfield et al., 1991). The bovine submandibular gland also shows predominance of O-acetylated derivatives (Schauer, 1982; Reuter et al., 1983; Corfield et al., 1991; Menghi et al., 1992).

On the basis of these data we considered it interesting to undertake a study about sialoglycoconjugates in the bovine sublingual gland. So, by means of histochemical techniques supported by lectin histochemistry and sialidase digestion we investigated *in situ* the occurrence of sialoglycoderivatives, their acetylation degree, the type of linkage and the acceptor sugars.

Offprint requests to: Prof. Daniela Accili, Dipartimento di Biologia M.C.A., Università di Camerino, Viale Aldo Moro 10, 62032 Camerino (MC), Italy

Materials and methods

Tissue processing

Female bovine sublingual glands gathered immediately after slaughter were fixed for 24 h at room temperature in Carnoy's fluid and postfixed in a 2% calcium acetate-4% paraformaldehyde solution (1:1) for 3 h. After dehydration with a graded series of ethanols, specimens were embedded in paraffin wax. Serial sections were cut at a thickness of 5 μm.

Lectins

The lectins used in this study are reported in Table 1. These lectins were horseradish peroxidase (HRP) labelled. PNA and DBA were purchased from Sigma Chemical Co. (St. Louis, Mo, USA) whereas LPA and SNA were obtained from USB (Cleveland, Ohio). LPA and SNA were selected for the direct visualization of sialic acid while PNA and DBA were chosen for the indirect detection, among lectins with similar nominal specificity, on the basis of previous findings (Accili et

al., 1990; Menghi et al., 1992).

Lectin staining procedures

Lectin staining was performed as previously reported (Menghi et al., 1989, 1991). Briefly, endogenous peroxidase activity was blocked by immersion in 0.3% hydrogen peroxide-methanol solution for 30 min and then sections were incubated for 30 min at room temperature with lectin-HRP conjugates (10-20 µg ml^{-1}). Peroxidase was developed with 3-3'-diamino-benzidine-hydrogen peroxide medium for 10 min (Graham and Karnovsky, 1966).

As controls for lectin specificity some sections were exposed in parallel to a mixture of the lectin-HRP conjugate and the appropriate hapten sugar at a concentration of 0.2-0.4M or immersed in a solution containing unconjugated lectin. In particular, for SNA labelling, N-acetylneuraminic acid, glycolylneuraminic acid, N-acetylgalactosamine and galactose were tested as inhibitory sugars.

Sialidase digestion procedure

Tissue sections adjacent to those exposed to each lectin were pretreated with sialidase digestion. Sialidase (neuraminidase type V from *Clostridium perfringens*, Sigma) digestion with the enzyme at a concentration of 0.5 U/ml, was performed for 16 h at 37° C in a solution of acetate buffer, pH 5.5, containing 10 mM $CaCl_2$ (Spicer and Warren, 1960). Control sections were incubated in enzyme-free buffer for the same period of time and under the same conditions (Plendl et al., 1989). Removal of sialic acid was verified on adjacent sections by loss of LPA and SNA staining after sialidase treatment. In addition, the specificity of sialidase digestion was confirmed by immersing some sections in sialidase solution and then in different lectins having affinity for various sugar residues.

Histochemical procedures

1.- Alcian blue (AB), pH 2.5, staining procedure was applied to tissues for 2 h at room temperature (Spicer et al., 1967).

2.- Periodate oxidation was performed using both 1mM aqueous periodic (1mM PO) and 44mM aqueous periodic acid (44mM PO) for 15 min at room temperature (Schauer, 1978).

3.- Saponification was carried out by 0.5% potassium hydroxide in 70% ethanol for 30 min at room temperature (Reid et al., 1978).

The following histochemical procedures were performed as sequential treatments:

a. AB (pH 2.5)
b. KOH/AB (pH 2.5)
c. PNA-DBA
d. 1mM-44mM PO/PNA-DBA
e. Sialidase/PNA-DBA
f. KOH/sialidase/PNA-DBA
g. 1mM-44mM PO/sialidase/PNA-DBA
h. 1mM-44mM PO/KOH/sialidase/PNA-DBA
i. KOH/1mM-44mM PO/sialidase/PNA-DBA

Results

Presence of terminal sialic acid residues was directly demonstrated by LPA (Fig. 1) and SNA (Fig. 2).

Table 1. Lectins employed and their nominal specificity.

LECTIN: LATIN NAME AND ACRONYM	BINDING SPECIFICITY	REFERENCE
Limulus polyphemus, LPA	Neu5Ac, Neu5Gc	Marchalonis and Edelman, 1968
Sambucus nigra, SNA	Neu5Acα2,6Gal/GalNAc	Shibuya et al., 1987
Arachis hypogaea, PNA	D-Galß1,3GalNAc	Lotan et al., 1975; Pereira et al., 1976
Dolichos biflorus, DBA	α-D-GalNAc	Hammarström et al., 1977

Neu5Ac: N-acetylneuraminic acid; Neu5Gc: N-glycolilneuraminic acid; Gal: galactose; GalNAc: N-acetylgalactosamine.

Table 2. PNA labelling with and without prior sialidase digestion and histochemical procedures.

	DEMILUNES	ACINI	STRIATED DUCTS	CARBOHYDRATE MOIETIES VISUALIZED
PNA	0	0-1	0	Detection of ß-Gal residues in a terminal non-reducing position
Sialidase/PNA	0	3-4	3-4[a,b]	Visualization of terminal dimer sialic acid-ß-Gal in which sialic acid does not present C_4 acetylated groups
KOH/sialidase/PNA	0	3-4	3-4[a,b]	Presence of sialic acid not C_4 O-acetylated linked to ß-Gal
1mM PO/sialidase/PNA	0	2-4	3-4[a,b]	Reactivity of sialic acid, with acyl substituents on the polyhydroxyl side chain, having ß-Gal as receptor
44mM PO/sialidase/PNA	0	1-4	1-2[a,b]	Occurrence of C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ O-acetylated sialic acid-(α2,3)-Gal

Results are based on a subjectively estimated scale ranging from 0 to 4 with 0 being unreactive and 4 being strongly reactive. [a]: luminal border; [b]: material inside the lumen.

Experiments to test the specificity of SNA binding were performed and it was noted that both sialidase pretreatment and incubation with SNA in presence of N-acetylneuraminic acid or N-glycolylneuraminic acid abolished SNA staining. Incubation with galactose (Fig. 3) and N-acetylgalactosamine (Fig. 4) decreased the SNA binding.

PNA and DBA staining patterns, obtained with and without prior sialidase treatment and the selected histochemical procedures, are listed in Tables 2, 3. Both tables provide an interpretation of carbohydrate moieties visualized by the different staining procedures and show the histological sites where the sugar sequences were found. The bovine monostomatic sublingual gland was composed of secretory end-pieces consisting of serous demilunes and mucous acini. The secretory tracts opened

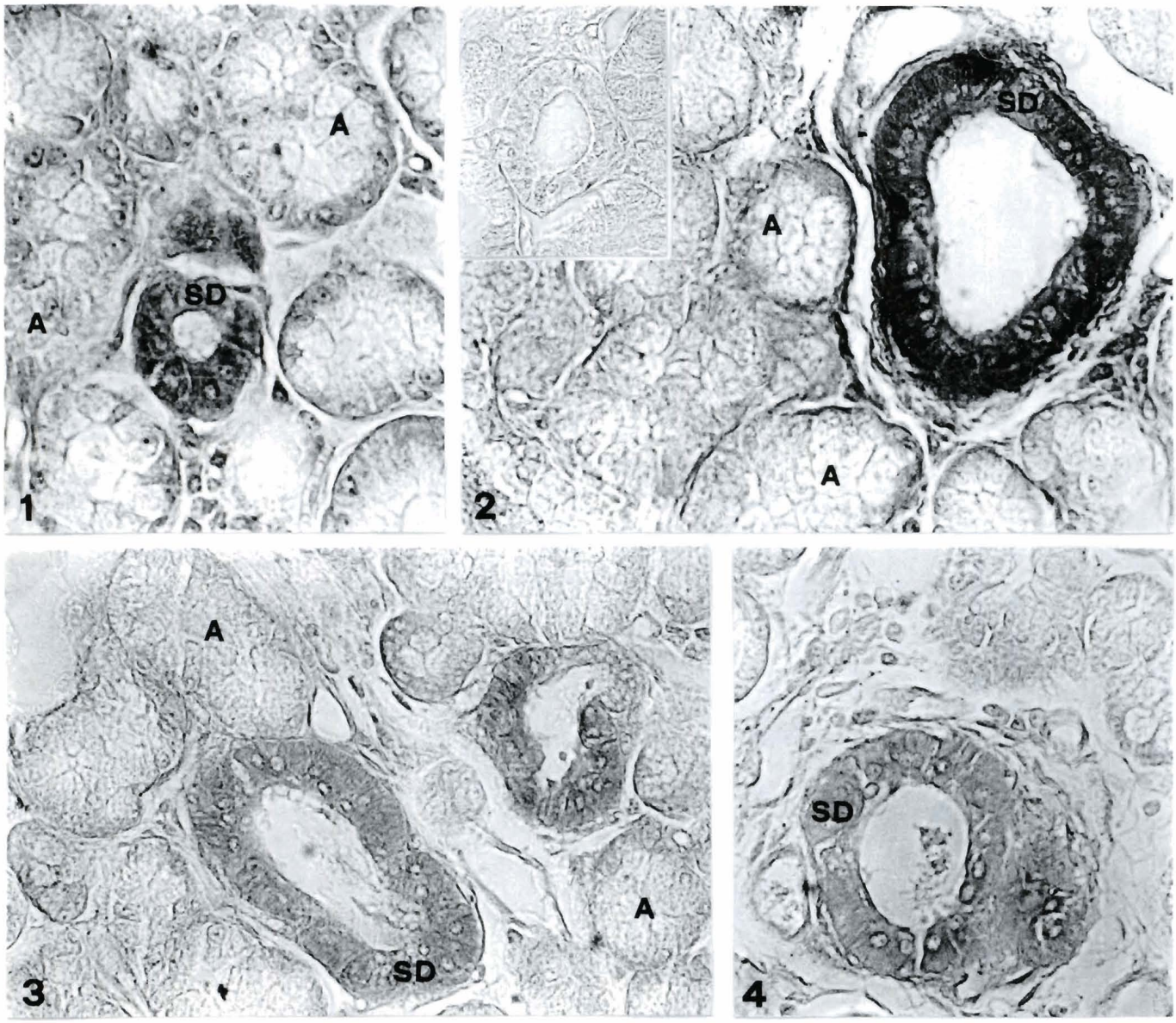


Fig. 1. LPA-HRP staining. Reaction is evident in striated duct (SD) cells. Acini (A). x 500

Fig. 2. SNA-HRP staining. Most SNA binding is present in the striated duct (SD) cells. Acini (A). x 500. Inset: Sialidase pretreatment completely removed SNA reactivity. x 280

Fig. 3. SNA-HRP staining. Galactose residues added to SNA-HRP solution result in a decreased SNA positivity. Striated ducts (SD). Acini (A). x 500

Fig. 4. SNA-HRP staining. Galactosamine also shows its inhibiting effect on SNA binding consisting in a reduction of affinity. Striated ducts (SD). x 500

into the initial part of a system of branched ducts.

Demilunes

The serous demilunar cells did not show sialylated molecules.

Acini

LPA (Fig. 1) and SNA (Fig. 2) showed a faint

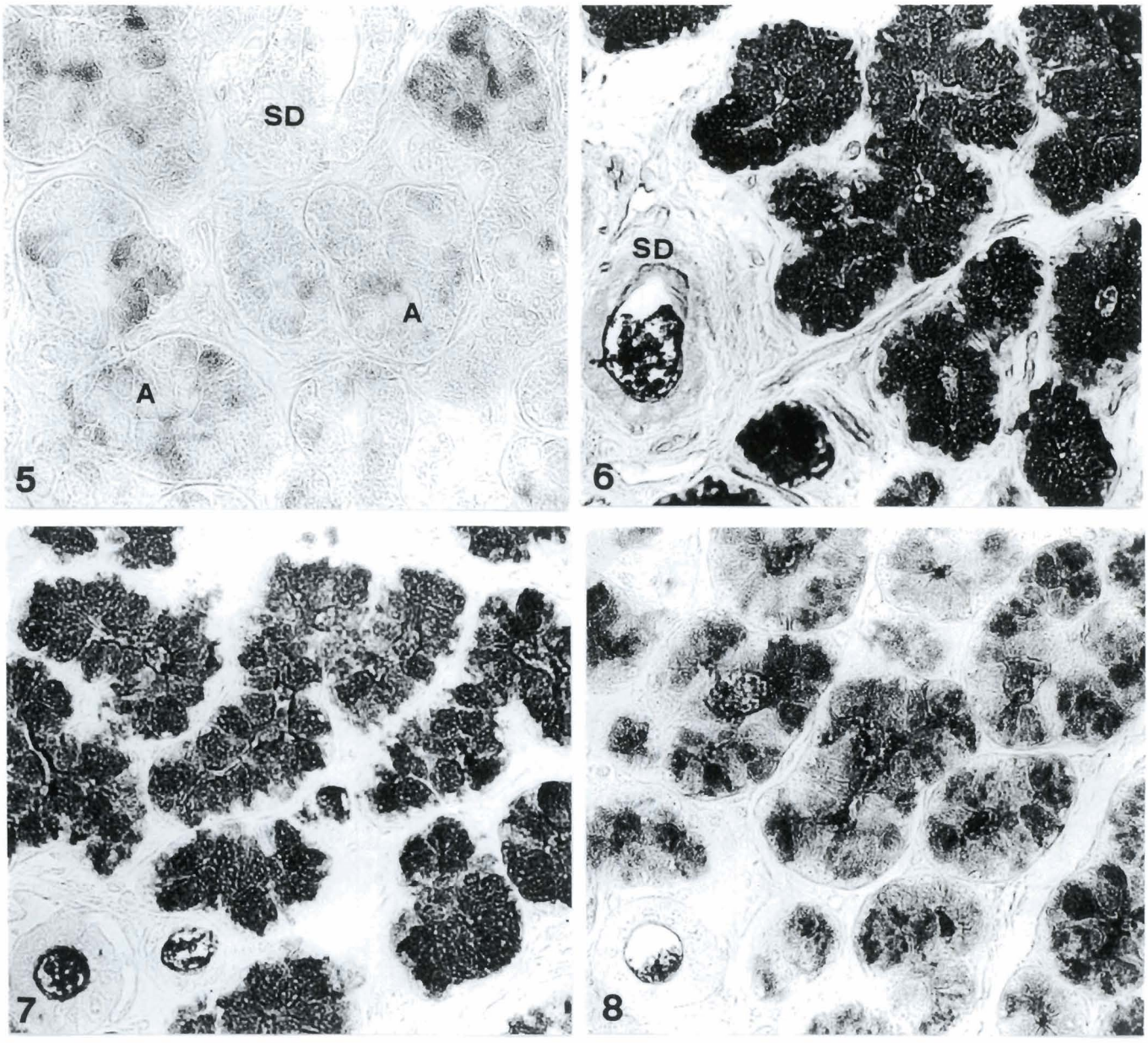


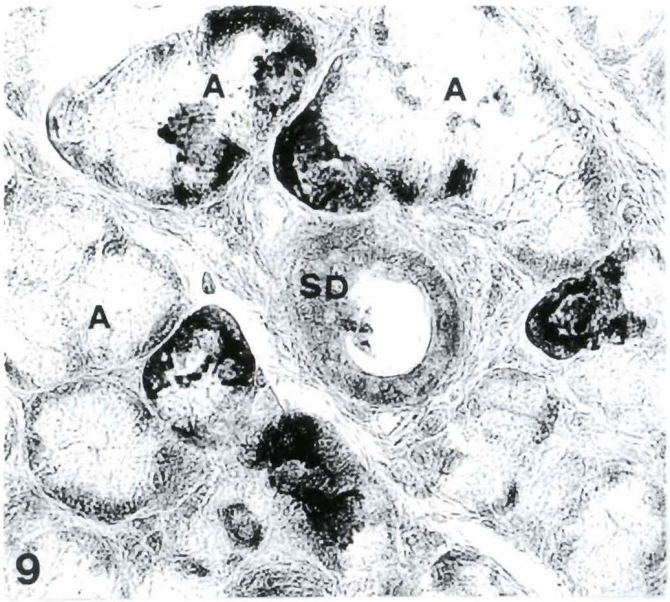
Fig. 5. Sialidase-free buffer PNA-HRP staining. Individual acinar cells (A) exhibit a variable content of terminal galactose. Demilunes and striated ducts (SD) are unreactive. x 450

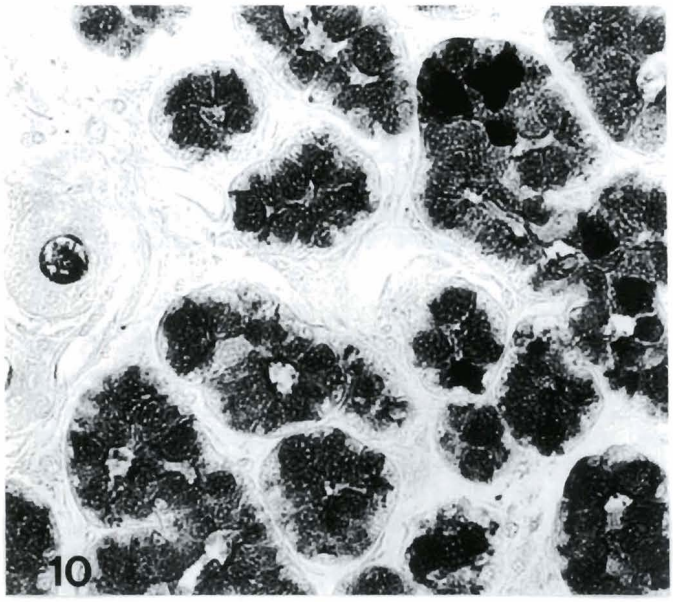
Fig. 6. Sialidase/PNA-HRP staining. Sialidase digestion imparts affinity for PNA in all acinar cells. Additional binding sites are present on the luminal border of striated ducts (SD) and on the luminal material. x 450

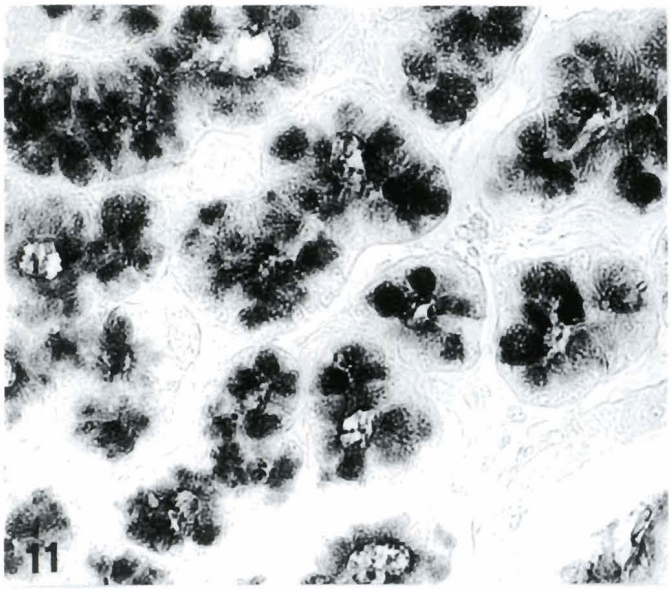
Fig. 7. 1mM PO/sialidase/PNA-HRP staining. Oxidation with 1mM periodate prior to sialidase only slightly decreases PNA staining after enzymatic treatment. x 450

Fig. 8. 44mM PO/sialidase/PNA-HRP staining. Pretreatment with 44mM periodate moderately reduces sialidase/PNA reactivity. x 450

reactivity restricted to the subnuclear region of a few acinar cells. Comparative findings from sequences employed indicated the presence of sialoderivatives in acinar cells (Figs. 5, 6, 9, 10). Almost all sialic acid residues linked to galactose, as reported in Table 2, contained O-acyl substituents which were not susceptible to oxidation by dilute periodic acid (Fig. 7). In addition, persisting PNA reactivity after 44 mM PO/sialidase/PNA sequence suggested that a percentage of these acetylated sialic acids contained C_9 acetyl groups (Dyer, 1956) and were linked to galactose in an alpha 2→3 configuration (Fig. 8).







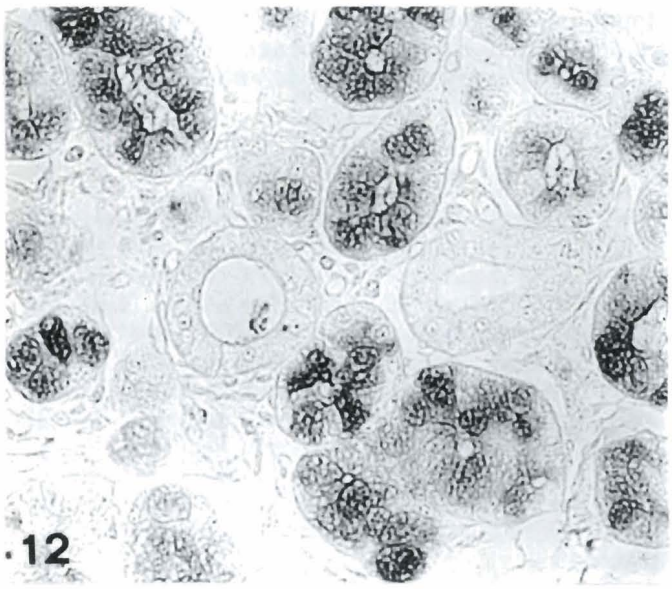


Fig. 9. Sialidase-free buffer/DBA-HRP staining. Acinar cells (A) vary in DBA reactivity. A group of stained cells is found adjacent to a group of unstained ones. Evident reaction is also present on ductal cells (SD) and material contained in the lumen. x 450

Fig. 10. Sialidase/DBA-HRP staining. After enzymatic cleavage of sialic acid residues, moderate to intense staining is only discovered in acinar cells. x 450

Fig. 11. 1mM PO/sialidase/DBA-HRP staining. This treatment reduces the reactivity of some acinar cells resulting in negative to intense staining. x 450

Fig. 12. 44mM PO/sialidase/DBA-HRP staining. Oxidation of sialic acid residues with 44mM periodic acid decreases acinar cell reactivity with respect to 1mM PO/sialidase/DBA sequence. x 450

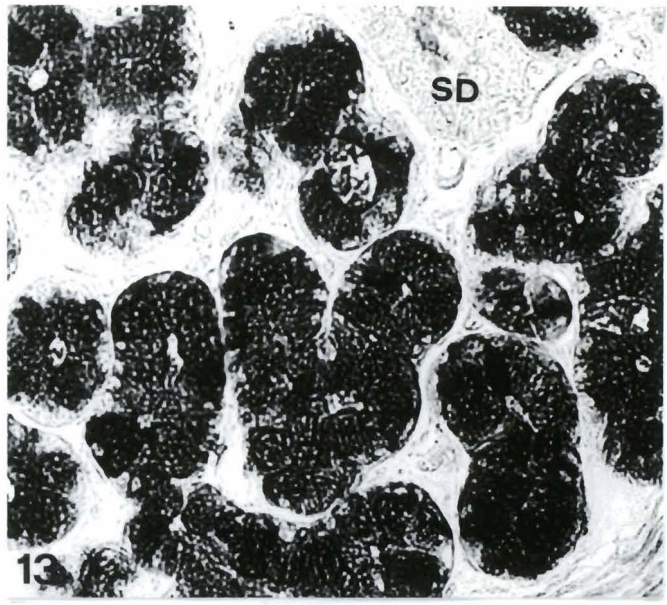
The sialoglycoconjugates having the terminal dimer sialic acid-N-acetylgalactosamine contained sialic acids with O-acetyl substituents (Table 3). In addition to sialic acid with C_7 and/or C_8 and/or C_9 acetylated groups as attested by 1mM PO/sialidase/DBA (Fig. 11) and 44mM PO/sialidase/DBA (Fig. 12), actual data indicated the presence of sialic acids O-acetylated at C_4 (Fig. 13) since KOH treatment increased sialidase digestion (Moshera and Pigman, 1975). The 1mM PO/KOH/sialidase/DBA and the 44mM PO/KOH/sialidase/DBA sequences indicated that C_4 sialoderivatives may contain more C_7 and/or C_8 (Fig. 14) and/or C_9 acetyl groups (Fig. 15). The absence of C_1 acetylated sialic acid, which analogously to C_4 could originate resistance to *Clostridium perfringens* sialidase, was supported by Alcian blue unchanged positivity after KOH treatment.

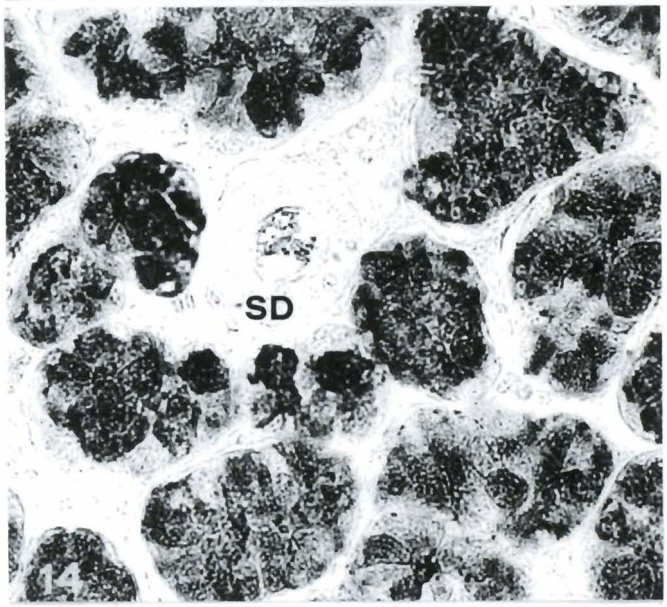
Striated ducts

Ductal cells stained uniformly with LPA (Fig. 1) and SNA (Fig. 2). Sialidase/PNA (Fig. 6) and sialidase/DBA (Fig. 10) bindings were distributed on the luminal border and material present inside the duct lumen. These sialoderivatives are considered to contain terminal sialic acid residues with O-acetyl groups. In particular, C_7 and/or C_8 acetylated sialic acid-($\alpha 2 \rightarrow 3$)-galactose (Fig. 7) and C_7 and/or C_8 acetylated sialic-($\alpha 2 \rightarrow 6$)-N-acetylgalactosamine (Fig. 11) were evidenced. In addition, resistance to 44mM periodate/sialidase/PNA (Fig. 8) or 44mM periodate/sialidase/DBA (Fig. 12) sequences indicated the presence of C_9 acetylated sialic acid linked to galactose and galactosamine respectively.

Controls

No staining was evidenced in sections exposed to PNA or DBA unconjugated lectins and to incubation with 0.2-0.4M galactose or 0.2-0.4M galactosamine in the PNA-HRP and DBA-HRP solutions. Oxidation with 1mM periodic acid had no effect on PNA and DBA binding, whereas pretreatment with 44mM periodic acid abolished PNA staining and decreased DBA labelling. Controls for efficacy and specificity of sialidase digestion were as expected. The immersion of sections in enzyme-free buffer solution resulted in unmodified binding. Deacetylation with KOH removed the reactivity due to periodate/sialidase/PNA or DBA treatments.





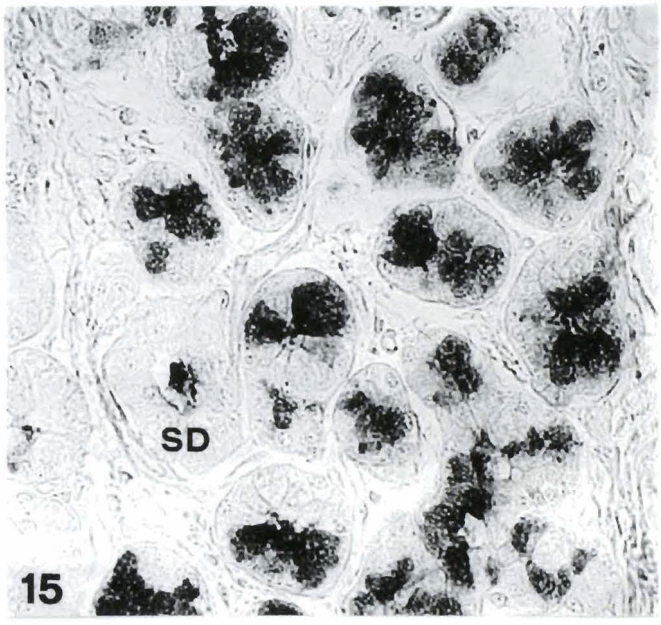


Fig. 13. KOH/sialidase/DBA-HRP staining. Saponification increases the DBA/sialidase-induced affinity. Striated duct (SD). x 450

Fig. 14. 1mM PO/KOH/sialidase/DBA-HRP staining. The basal region of some acinar cells is unstained with the sialidase/DBA-HRP sequence following 1mM PO/KOH pretreatment. Striated duct (SD). x 450

Fig. 15. 44mM PO/KOH/sialidase/DBA-HRP staining. A population of DBA-positive acinar cells is stained after the 44mM PO/KOH/sialidase/DBA-HRP sequence. Striated duct (SD). x 450

Table 3. DBA labelling with and without prior sialidase digestion and histochemical procedures.

	DEMILUNES	ACINI	STRIATED DUCTS	CARBOHYDRATE MOIETIES VISUALIZED
DBA	0	0-3	1-2	Glycosylated receptors containing α-GalNAc in a terminal exposed position
Sialidase/DBA	0	2-4	1-2[a,b]	Visualization of moieties having terminal dimers with the sequence sialic acid-GalNAc in which sialic acid does not present C_4 acetylated radicals
KOH/sialidase/DBA	0	3-4	1-2[a,b]	Detection of sialoglycoconjugates containing sialic acid, with and without C_4 acetyl groups, linked to GalNAc
1mM PO/sialidase/DBA	0	0-4	0-2[b]	Presence of terminal sialic acid, with O-acetylated polyhydroxyl side chain, bound to penultimate GalNAc
44mM PO/sialidase/DBA	0	0-2	0-1[b]	Reactivity of C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$ acetylated sialic acid linked to the underlying GalNAc
1mM PO/KOH/sialidase/DBA	0	2-3	0-2[b]	Localization of sialic acid, with and without C_4 acetyl groups in addition to O-acetylated side chain, having GalNAc as receptor
44mM PO/KOH/sialidase/DBA	0	0-3	0-2[b]	Terminal dimer sialic acid-GalNAc with sialic acid acetylated both in C_4 and in C_9 and/or $C_{7,9}$ and/or $C_{8,9}$ and/or $C_{7,8,9}$

Results are based on a subjectively estimated scale ranging from 0 to 4 with 0 being unreactive and 4 being strongly reactive. [a]: luminal border; [b]: material inside the lumen.

Discussion

The investigation of sialoglyconjugates in the bovine sublingual gland was carried out starting from peroxidase-conjugated LPA and SNA which showed overlapping results at ductal cell level. Both lectins were chosen owing to their ability to visualize sialic acid residues by direct binding, as already reported (Yamada and Shimizu, 1979; Muresan et al., 1982; Shibuya et al., 1987; Taatjes et al., 1988; Oulhaj et al., 1993) but neither LPA nor SNA were of complete acceptance as histochemical reagents. Indeed, LPA sometimes gave negative results apparently because of its very large size and relative instability (Goldstein and Poretz, 1986).

For SNA the question was the specificity. The SNA reactive oligosaccharide sequences Neu5Ac(α2,6)Gal/GalNAc were tested in Kurloff cells of guinea pig thymus (Oulhaj et al., 1993), in bovine and sheep submandibular gland, in rat liver and kidney (Taatjes et al., 1988) and in rat small intestine during postnatal development (Taatjes and Roth, 1990) and at these sites the application of SNA evidenced certain limitations. To also test in the bovine sublingual gland the possibility of interference in SNA binding, accurate controls were performed. The enzymatic removal of sialic acid and the incubation of SNA in presence of N-acetylneuraminic acid or N-glycolylneuraminic acid resulted in the abolition of SNA reactivity, but galactose and N-acetylgalactosamine residues also had their inhibitory effect according to agglutination assay with trypsin-treated human blood-group-A red blood cells reported by Broekaert et al., (1984) which showed that lactose, N-acetylgalactosamine and galactose were the saccharide inhibitors of elder bark agglutinin.

In order to by-pass these problems and to look for new information which the sole use of specific lectins cannot generally yield, we combined periodate oxidation, sialidase digestion and staining with PNA- and DBA-HRP conjugates. Thus, it was possible to identify the penultimate sugars present in the bovine sublingual sialoglycoconjugates, the linkage relationship, the occurrence and the type of O-acetylated groups in sialic acids. The main acquisitions are as follows. In the first instance, we evidenced the presence of two terminal dimers, i.e. sialic acid-galactose and sialic acid-N-acetylgalactosamine at acinus level on the basis of increased PNA and DBA stainings after sialidase digestion, whereas no reaction was found in ductal cells as expected from LPA and SNA positivity. This non-overlap of results is difficult to explain. Probably, the lack of LPA and SNA affinity in acinar cells, which were seen to contain sialic acid from the strong positivity of PNA and DBA after sialidase pre-treatment, could be due to difficult internalization and binding of the two lectins towards secretory sialoglyco-derivatives which are highly packaged. On the other hand, LFA, an additional sialic acid recognizing lectin, also showed a similar discrepancy in ciliated cells and vascular endothelial cells of the hamster trachea (Schulte et al., 1984). As regards the lack of PNA and DBA reactivity after sialidase treatment in ductal LPA and SNA positive cells, the possible hypothesis advanced by us is that the enzymatic cleavage of sialic acid would be likely to have led to changes of the lectin receptors with consequent negative binding.

The second indication was that almost all terminal sialic acids linked to penultimate galactose were in an alpha 2→3 configuration, since 44mM periodate moderately reduced sialidase-PNA staining. Indeed, the α2→3 linkage, which was relatively rigid, when compared to the free rotation of the α2→6 linkage (Montreuil, 1980), prevented the penultimate galactose

from oxidation, thus resulting able to bind PNA lectin.

Thirdly, a very relevant distribution of O-acetylated sialic acids which are not oxidized by mild periodate was found (McLean et al., 1970). The bovine sublingual sialoglyconjugates exhibited a noticeable amount of 9-O-acetylated sialic acids linked to both galactose and N-acetylgalactosamine which are not susceptible to oxidation by strong periodic acid oxidation (Dyer, 1956; Schauer, 1978); this datum is very different from that observed in the bovine submandibular gland where 25-33% of sialic acids (Pigman, 1977) consist of 9-O-acetylated residues linked to N-acetylgalactosamine only (Menghi et al., 1992). These findings are in accordance with earlier biochemical results which have detected an amount of 15.13% sialic acids with the thiobarbituric method in contrast to a value of 6.58% estimated by the resorcinol assay (Menghi et al., 1990). In addition, the KOH/sialidase/DBA and KOH/Alcian blue sequences demonstrated the presence of C_4 acetylated sialic acids linked to N-acetylgalactosamine residues. Generally, the heterogeneity in morphological cytoarchitecture and secretory products of various salivary glands has been correlated with a different biological significance of these organs (Pinkstaff, 1980; Hand, 1986). Hypotheses about their glycoconjugate involvement in protective function have also been advanced. Indeed, one might speculate that the presence in bovine sublingual gland of C_4 acetylated sialic acids would play a role in defensive mechanisms. Accordingly, the 4-O-acetyl group prevents influenza C virus from recognizing its receptor determinant, an effect which corresponds to the hindered action of sialidases and N-acylneuraminate lyase on 4-O-acetylated sialic acids (Hanaoka et al., 1989; Zimmer et al., 1992).

Acknowledgements. The authors wish to thank Ms. S. Cammertoni and Mr. S. Riccioni for the assistance in the histochemical stainings. This work was supported by grants (40% and 60%) from M.U.R.S.T., Italy.

References

Accili D., Menghi G., Scocco P. and Bondi A.M. (1990). Lectin histochemistry for differentiating glycoconjugates secreted by the bovine submandibular gland. 53 º Congr. Unione zoologica italiana, ed. U.Z.I. Palermo. p 416.

Broekaert W.F., Nsimba-Lubaki M., Peeters B. and Peumans W.J. (1984). A lectin from elder (*Sambucus nigra* L.) bark. Biochem. J. 221, 163-169.

Corfield A.P., Corfield C.D.A., Veh R.W., Wagner S.A., Clamp J.R. and Schauer R. (1991). Characterization of the major and minor mucus glycoproteins from bovine submandibular gland. Glycoconjugate J. 8, 330-339.

Dall'Olio F., Malagolini N. and Serafini-Cessi F. (1992). Enhanced CMP-NeuAc:Galß1,4GlcNAc-R α2,6 sialyltransferase activity of human colon cancer xenografts in athymic nude mice and of xenograft-derived cell lines. Int. J. Cancer 50, 325-330.

Devine P.L., Clark B.A., Birrell G.W., Layton G.T., Ward B.G., Alewood P.F. and McKenzie I.F.C. (1991). The breast tumor-associated epitope defined by monoclonal antibody 3E1.2 is an O-linked mucin carbohydrate containing N-glycolylneuraminic acid. Cancer Res. 51, 5826-5836.

Dyer J.R. (1956). Use of periodate oxidations in biochemical analysis. In: Methods of Biochemical Analysis. Vol. III. Glick D. (ed). Interscience. New York. pp 111-123.

Goldstein I.J. and Poretz R.D. (1986). Isolation and chemical properties of lectins. In: The lectins. Liener I.E., Sharon N. and Goldstein I.J. (eds). Academic Press. New York. pp 233-247.

Graham R.C. Jr. and Karnovski M.J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14, 291-302.

Hammarström S., Murphy L.A., Goldstein I.J. and Etzler M.E. (1977). Carbohydrate binding specificity of four N-acetyl-D-galactosamine «specific» lectins: Helix pomatia A hemagglutinin, soybean agglutinin, limabean lectin, and Dolichos biflorus lectin. Biochemistry 16, 2750-2755.

Hanaoka K. Pritchett T.J., Takasaki S., Kochibe N., Sabesan S., Paulson J.C. and Kobata A. (1989). 4-O-acetyl-N-acetylneuraminic acid in the N-linked carbohydrate structures of equine and guinea pig α_2-macroglobulins, potent inhibitors of influenza virus infection. J. Biol. Chem. 264, 9842-9849.

Hand A.R. (1986). Salivary glands. In: Orban's oral histology and embryology. 10th ed., chap. 10. Bhaskar S.N. (ed). CU Mosby. St. Louis, Mo. pp 328-360.

Lotan H., Skutelsky E., Danon D. and Sharon N. (1975). The purification, composition and specificity of the anti-T lectin from peanut *(Arachis hypogaea).* J. Biol. Chem. 250, 8518-8523.

Mancini G.M.S., Verheiejn F.W., Beerens C.E.M.T., Renlund M. and Aula P. (1991). Sialic acids storage disorders: observations on clinical and biochemical variation. Dev. Neurosci. 13, 327-330.

Marchalonis J.J. and Edelman G.M. (1968). Isolation and characterization of a hemagglutinin from *Limulus polyphemus.* J. Mol. Biol. 32, 453-465.

McLean R.L., Suttajit M., Beidler J. and Winzler R.T. (1970). N-acetylneuraminic acid analogues. I. Preparation of the 8-carbon and 7-carbon compounds. J. Biol. Chem. 246, 803-809.

Menghi G., Accili D., Bondi A.M. and Gabrielli M.G. (1989). Enzymatic degradation and quantitative lectin labeling for characterizing glycoconjugates which act as lectin acceptors in cat submandibular gland. Histochemistry 90, 331-338.

Menghi G., Piciotti D., Luciani S. and Timperio A.M. (1990). Anti-coagulant activity with dose-related response of glycoconjugates from bovine sublingual gland. Cell Mol. Biol. 36, 557-564.

Menghi G., Ottaviani E., Accili D. and Bolognani Fantin A.M. (1991). Identification of muramyl derivatives in Mollusca Gastropoda tissue. Histochemistry 96, 209-216.

Menghi G., Accili D., Scocco P. and Materazzi G. (1992). Sialoglyco-derivatives of bovine submandibular gland identified in situ by histochemical techniques combined with lectins. Histochemistry 97, 397-403.

Montreuil J. (1980). Primary structure of glycoproteinglycans: basis for the molecular biology of glycoproteins. Adv. Carbohydr. Chem. Biochem. 37, 157-213.

Moshera J. and Pigman W. (1975). The isolation and characterization of rat sublingual mucus-glycoprotein. Carbohydr. Res. 40, 53-67.

Muresan V., Iwanij V., Smith Z.D.J. and Jamieson J.D. (1982). Purification and use of limulin: A sialic acid-specific lectin. J.

Histochem. Cytochem. 30, 938-946.

Oulhaj N., Letaïef S-E., Landemore G. and Izard J. (1993). Glycoconjugates with Neu5Ac(α2,6)Gal(ß1,4)GlcNAc sequences: a selective lectin-histochemical property of Kurloff cells in guinea pig thymus. Cell Tissue Res. 271, 69-75.

Pereira M.E.A., Kabat E.A., Lotan R. and Sharon N. (1976). Immunochemical studies on the specificity of the peanut *(Arachis hypogaea)* agglutinin. Carbohydr. Res 51, 107-118.

Pigman W. (1977). Submandibular and sublingual glycoproteins. In: The glycoconjugates. Vol. 1. Horowitz M.I. and Pigman W. (eds). Academic Press. New York. pp 137-152.

Pinkstaff C.A. (1980). The cytology of salivary glands. Int. Rev. Cytol. 63, 141-261.

Plendl J., Schönleber B., Schmahl W. and Schumacher V. (1989). Comparison of the unmasking of lectin receptors by neuraminidase and by enzyme-free buffer alone. J. Histochem. Cytochem. 37, 1743-1744.

Reid P.E., Culling C.F.A. and Dunn W.L. (1978). A histochemical method for the identification of 9-0-acyl sialic acids. An investigation of bovine submaxillary gland and intestinal mucins. J. Histochem. Cytochem. 26, 187-192.

Reuter G., Pfeil L., Stol S., Schauer R., Kamerling J.P., Versluis C. and Vliegenthart J.F.G. (1983). Identification of new sialic acids derived from glycoproteins of bovine submandibular gland. Eur. J. Biochem. 134, 139-143.

Reutter W., Köttgen E., Bauer C. and Gerok W. (1982). Biological significance of sialic acids. In: Sialic acids. Chemistry, metabolism and function. Cell Biology Monographs. Vol. 10. Schauer R. (ed). Springer. Wien. pp 263-305.

Schauer R. (1978). Characterization of sialic acids. In: Methods in enzymology. Vol. 50. Ginsburg V. (ed). Academic Press. New York. pp 65-89.

Schauer R. (1982). Chemistry, metabolism and biological functions of sialic acid. Adv. Carbohydr. Chem. Biochem. 40, 131-234.

Schauer R. (1987). Analysis of sialic acids. In: Methods in enzymology. vol. 138. Ginsburg V. (ed). Academic Press. New York. pp 132-161.

Schulte B.A. and Spicer S.S. (1985). Histochemical methods for characterizing secretory and cell surface sialoglycoconjugates. J. Histochem. Cytochem. 33, 427-438.

Schulte B.A., Spicer S.S. and Miller R.L. (1984). Histochemical localization of sialoglycoconjugates which a sialic acid-specific lectin from the slug *Limax flavus*. Histochem. J. 16, 1125-1132.

Schulte B.A., Spicer S.S. and Miller R.L. (1985). Lectin histochemistry of secretory and cell-surface glycoconjugates in the ovine submandibular gland. Cell Tissue Res. 240, 57-66.

Shibuya N., Goldstein I.J., Broekaert W.F., Nsimba-Lubaki M., Peeters B. and Peumans W.J. (1987). The elderberry (*Sambucus nigra* L.) bark lectin recognizes the Neu5Ac (α2,6)GalNAc sequence. J. Biol. Chem. 262, 1596-1601.

Spicer S.S. and Warren L. (1960). The histochemistry of sialic acid containing mucoproteins. J. Histochem. Cytochem. 8, 135-137.

Spicer S.S., Horn R.G. and Leppi T.J. (1967). Histochemistry of connective tissue mucopolysaccharides. In: The connective tissue. Wagner B.M. and Smith D.E. (eds). Williams & Wilkins. Baltimore. pp 251-303.

Taatjes D.J. and Roth J. (1990). Selective loss of sialic acid from rat small intestinal epithelial cell during postnatal development: demonstration with lectin-gold techniques. Eur. J. Cell Biol. 53, 255-266.

Taatjes D.J., Roth J., Peumans W. and Goldstein I.J. (1988). Elderberry bark lectin-gold techniques for the detection of Neu5Ac (α2,6) Gal/GalNAc sequences: applications and limitations. Histochem. J. 20, 478-490.

Werner G., Addicks K., Fricke U., Klaus W., Sarram M. and Gielen W. (1991). Sialic acid removal modules the miocardial and vascular activity of calcium channel ligands. Biochem. Pharmacol. 42, S77-S87.

Yamada R. and Shimizu S. (1979). The use of peroxidase-labelled *Limulus polyphemus* agglutinin for the histochemistry of sialic acid-containing glycoproteins in light microscopy. Histochem. J. 11, 457-471.

Zimmer G., Reuter G. and Schauer R. (1992). Use of influenza C-virus for detection of 9-O-acetylated sialic acids on immobilized glycoconjugates by esterase activity. Eur. J. Biochem. 204, 209-215.

Accepted June 28, 1994