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Elderberry bark lectin–gold techniques for the detection of Neu5Ac (α2,6) Gal/GalNAc sequences: applications and limitations

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Summary

The lectin from the elderberry (*Sambucus nigra* L.) bark, shown to recognize the sequence neuraminic acid (α2,6) galactose/*N*-acetylgalactosamine, was applied for detecting binding sites in Lowicryl K4M sections by light and electron microscopy. The lectin was used either directly complexed to colloidal gold or in a two-step cytochemical affinity technique. The lectin–gold complex proved to be superior and thus was extensively tested on rat liver, kidney and hepatoma cells as well as on sheep and bovine submandibular glands. Controls to establish specificity of lectin–gold binding included sugar and glycoprotein inhibition tests and enzymic removal of sialic acid. In agreement with biochemical data demonstrating the potentiating effect of sialic acid on the binding of the lectin to oligosaccharides, enzymic removal of sialic acid from liver sections resulted in abolition of lectin staining. However, in the submandibular glands, neuraminidase pretreatment of the sections had no effect on the subsequent lectin–gold binding. In rat kidney some structures became negative while others retained the lectin–gold staining due to binding to penultimate *N*-acetylgalactosamine exposed after sialic acid removal. In line with this, spot blot analysis demonstrated that the lectin–gold complex reacted with both fetuin and asialofetuin. Taken together, these results suggest that, for cytochemical staining, the sialic acid and the galactose/*N*-acetylgalactosamine lectin combining subsites of *Sambucus nigra* L. lectin are equally reactive with cellular glycoconjugates and that neuraminidase predigestion of tissue sections is of utmost importance to ensure specificity of staining for the sequence neuraminic acid (α2,6) galactose/*N*-acetylgalactosamine.

Introduction

It is well established that sialic acid residues present on glycoproteins and glycolipids are involved in a variety of biological interactions (for review see Schauer, 1982). Such interactions may be governed by the chemical form as well as the type of linkage in which sialic acid is present, as illustrated by the binding of different strains of influenza virus to host cells (Rogers *et al.*, 1985,1986). Thus, it would be of value to obtain information about the tissue distribution of the various forms of sialic acid. In comparison to classical histochemical stains, monoclonal antibodies and lectins seem to provide such a degree of specificity. Although monoclonal antibodies specifically interacting with 9-*O*-acetylated neuraminic acid (Cheresh *et al.*, 1984) or with neuraminic acid in α2,6- (Hakomori *et al.*, 1983), α2,3- (Tai *et al.*, 1987) and α2,8- (Frosch *et al.*, 1985) linkages have been described, only the last named has been used for immunohistochemistry (Roth *et al.*, 1987). On the other hand, sialic acid-specific lectins isolated from *Limulus polyphemus* (Marchalonis & Edelman, 1968) or *Limax flavus* (Miller et al., 1982) have been widely used for cytochemical labelling (Mazzuca *et al.*, 1977; Yamada & Shimizu, 1979; Muresan *et al.*, 1982a, b; Roth *et al.*, 1984, 1985, 1986; Charest & Roth, 1985; Roth & Taatjes, 1985; Hedman *et al.*, 1986). However, both lectins have a rather broad reactivity with the different forms of sialic acid (Miller *et al.*, 1982; Ravindranath *et al.*, 1985) and the *Limulus polyphemus* lectin has, in addition, a binding site for phosphorylcholine (Mohan *et al.*, 1983). Wheatgerm agglutinin, although often used in histochemical studies is neither a specific nor sensitive probe for

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sialic acid, but rather for oligomers of *N*-acetylglucosamine (Goldstein & Poretz, 1986). Indeed, the basis for the weak binding to sialic acid is the result of structural similarities with *N*-acetylglucosamine (Goldstein & Poretz, 1986) which is also responsible for the weak binding of wheatgerm agglutinin to *N*-acetylgalactosamine (Bhavanandan & Katlic, 1979; Peters *et al.*, 1979).

Recently, lectins with a narrow specificity for sialic acid have been purified: the *Cancer antennaria* lectin recognizing 9-*O*-, and to a lesser extent 5-*O*-acetylated neuraminic acid (Ravindranath *et al.*, 1985), the *Achatina fulica* lectin reacting with 9-*O*-acetylated neuraminic acid (Mandal & Basu, 1987) and the *Sambucus nigra* L. (SNA, elderberry bark) lectin recognizing neuraminic acid α2,6-linked to galactose (Gal) or *N*-acetylgalactosamine (GalNAc) (Shibuya *et al.*, 1987a). Here, we report the preparation of an elderberry bark lectin–gold (SNA–g) complex and its use for light and electron microscopic postembedding labelling as well as detection of nitrocellulose-immobilized glycoproteins. Furthermore, we have used SNA in conjunction with fetuin–gold complex in a two-step cytochemical affinity technique.

Materials and methods

REAGENTS

The lectin from *Sambucus nigra* L. (SNA) was isolated and affinity purified as previously reported (Broekaert *et al.*, 1984; Shibuya *et al.*, 1987a). The lectin from *Limax flavus* was purchased from Calbiochem Behring (San Diego, CA, USA) and the *Helix pomatia* lectin from Pharmacia Fine Chemicals (Uppsala, Sweden). Tetrachloroauric acid, ascorbic acid, hydroquinone, paraformaldehyde, lactose and ammonium chloride were from Merck (Darmstadt, FRG); silver lactate, carbowax 20M and glutaraldehyde (25%, vacuum distilled) from Fluka (Buchs, Switzerland); poly-L-lysine (M_r 560 000), *N*-acetylneuraminic acid, galactose, glucose, galactosamine, *N*-acetylgalactosamine, fetuin, orosomucoid, bovine serum albumin and neuraminidase (type X from *Clostridium perfringens*) from Sigma (St Louis, MO, USA); and nitrocellulose sheets from Millipore (Bedford, MA, USA). Neu5Ac(α2,3) lactose and Neu5Ac(α2,6) lactose were the generous gift of Dr R. Schauer (University of Kiel) and asialoorosomucoid of Dr J. C. Paulson (University of California, Los Angeles). The asialofetuin was available from previous studies. All other reagents were of the highest purity available.

PREPARATION OF THE SNA–G COMPLEX

A polydispersed colloidal gold (5–12 nm) was prepared according to Stathis & Fabrikanos (1958). For complex formation with SNA, the pH of the colloidal gold was adjusted to 6.0–6.2. The minimal amount of lectin needed for stabilization of a given volume of colloidal gold was determined by the salt flocculation test (Roth & Binder, 1978). For complex formation, 400 μg SNA dissolved in double-distilled water was mixed with 10 ml colloidal gold, followed by the addition of 1 ml 1% polyethylene glycol. The crude SNA–gold complex was centrifuged at 105 000 *g* (r_{max}) for 45 min at 4° C and the sediment was resuspended with phosphate-buffered saline (PBS; 0.01 M phosphate buffer, pH 7.4, and 0.15 M sodium chloride) containing 2 mg ml^{-1} polyethylene glycol. Density gradient centrifugation on a 10–30% sucrose gradient was performed according to Slot & Geuze (1981).

TISSUE PROCESSING

Sheep and bovine submandibular glands and monolayer cultures of a diethylnitrosamine-induced rat hepatoma (Roth *et al.*, 1975) were fixed by immersion in 3% formaldehyde (freshly prepared from paraformaldehyde)–0.1% glutaraldehyde in PBS for 2 h and 30 min, respectively, at room temperature. Rat liver and kidney were fixed by vascular perfusion with 3% formaldehyde–0.1% glutaraldehyde as previously described (Roth *et al.*, 1985). After two brief rinses with PBS, all tissue and cells were treated with 50 mM ammonium chloride in PBS for 1 h, rinsed in PBS, and stored in PBS overnight at 4° C. Low-temperature embedding in Lowicryl K4M was carried out as previously described (Roth *et al.*, 1981).

CYTOCHEMICAL LABELLING PROTOCOLS

Light microscopy

Semithin (1μm) sections were mounted on poly-L-lysine-coated glass slides and dried at 45° C overnight. For labelling, sections were first covered with PBS for 5 min. The excess PBS was then drained away, and the sections incubated with the SNA–g complex for 45 min at room temperature. The SNA–g complex was diluted with PBS to yield a final optical density (OD) at 525 nm of 1.1. Subsequently, the sections were rinsed with PBS (2 × 5 min), covered with 1% glutaraldehyde in PBS for 20 min, rinsed with PBS (2 × 2 min), distilled water (2 × 2 min) and air dried. Finally, the gold signal was amplified with the photochemical silver reaction described previously (Taatjes *et al.*, 1987a).

Binding sites for SNA in tissue sections were also visualized by a two-step affinity technique with fetuin–gold complexes as second step reagent. Semithin sections from bovine submandibular gland were covered with PBS for 5 min, followed by incubation with SNA (100 or 250 μg ml^{-1}) in PBS for 45 min at room temperature. After two rinses with PBS for 5 min each, the sections were covered with fetuin–gold complex ($OD_{525 nm}$ = 0.4) for 30 min at room temperature. The fetuin–gold complex was prepared with ascorbic acid gold as described previously (Roth *et al.*, 1984). Sections were rinsed, fixed with glutaraldehyde and subjected to photochemical silver reaction as above.

Electron microscopy

Ultrathin sections on nickel grids coated with parlodion-carbon were floated on a droplet of PBS for 5 min, followed by transfer to a droplet of SNA–g ($OD_{525 nm}$ = 0.5–0.7) and incubated for 45 min at room temperature. Afterwards, the grids with the attached sections were rinsed with PBS (2 × 5 min) and distilled water and dried. Sections were counterstained with 3% aqueous uranyl acetate (5 min) and lead acetate (45 s).

CONTROLS

Various saccharides and glycoproteins as listed in the reagent section were used for preincubation (30 min) of the SNA–g, followed by its use for incubation as described above. Tissue sections were also pretreated with neuraminidase (4 units ml^{-1} for 6–15 h at 37° C for the removal of sialic acid residues, or in acetate buffer alone (6–15 h at 37° C).

DETECTION OF NITROCELLULOSE-IMMOBILIZED GLYCOCONJUGATES

Dot blots

The following (glyco)proteins (1 μg in 1 μl) were spotted onto nitrocellulose strips: fetuin, asialofetuin, orosomucoid, asialoorosomucoid and bovine serum albumin (BSA). After air drying, the strips were saturated with 1% BSA in PBS for 1 h at room temperature to reduce non-specific binding, followed by incubation with SNA–g ($OD_{525\,nm}$= 1.1) for 45 min. The strips were then washed with PBS containing 1% BSA and 0.1% Tween 20 (2 × 5 min) and distilled water (2 min).

Identical blocked strips were incubated with the *Limax flavus* lectin (100 μg ml^{-1}, 45 min), rinsed with PBS (2 × 5 min), incubated for 30 min with fetuin–gold complex (10 nm gold particles, diluted with PBS containing 1% BSA, 0.075% Triton X–100 and 0.075% Tween 20 to an $OD_{525\,nm}$ of 0.3) and rinsed with PBS containing 1% BSA and 0.1% Tween 20 (2 × 5 min) and distilled water (2 min).

Lectin overlay on nitrocellulose transfers

Homogenates were prepared from rat liver as well as monolayer cultures of hepatoma cells and stored at −20° C. SDS–polyacrylamide gel (10%) electrophoresis and transfer to nitrocellulose was carried out according to standard protocols (Laemmli, 1970; Towbin *et al.*, 1979). Nitrocellulose strips were blocked with 1% BSA in PBS for 30 min up to 16 h. This was followed by incubation with SNA–g complex diluted to give an $OD_{525\,nm}$ of 1.1 with PBS or PBS containing 0.5% gelatin for 45 min at room temperature. Strips were then rinsed with PBS containing 1% BSA and 0.1% Tween 20 (3 times 5 min each) and distilled water (2 times 2 min each). In some cases silver amplification was done as described above.

In control incubations the SNA–g complex was preincubated with 100 μg ml^{-1} fetuin or nitrocellulose strips were pretreated with neuraminidase as described above.

Other lectin–gold techniques

A *Helix pomatia* lectin–gold complex was prepared using 14-nm gold particles (Roth, 1983) and applied to semithin sections as described previously (Taatjes *et al.*, 1987a). The *Limax flavus* lectin/fetuin–gold technique (Roth *et al.*, 1984) was performed on semithin sections with subsequent silver amplification (Taatjes *et al.*, 1987a).

Results

In order to establish the applicability of the SNA–g complex as well as the SNA/fetuin–gold complex technique for the cytochemical demonstration of the Neu5Ac(α2,6)Gal/GalNAc sequence, we chose selected tissues known to contain glycoconjugates possessing this disaccharide unit: submandibular glands, liver, hepatoma cells and kidney.

Light and electron microscopy

Incubation of semithin sections from rat kidney with SNA–g followed by silver amplification resulted in intense staining of glomeruli and all tubular elements (Fig. 1). In the cortical portion of thick ascending limb of Henle', positive and negative cells were observed as previously reported using the *Limax flavus* lectin (Roth & Taatjes, 1985).

The mucus of the sheep submandibular gland is characterized by the presence of the disaccharide structure Neu5Ac(α2,6)GalNAc linked to the amino acids serine or threonine (Hill *et al.*, 1977) and thus we chose to investigate this tissue with the SNA. As shown in Fig. 2, intense staining in semithin sections was present over the mucus of the mucous cells and less intense staining over the secretion granules of the serous cells. The epithelia of the excretory ducts exhibited intense staining along the entire cell surface. These observations were corroborated at the electron microscope level. The mucus droplets showed diffuse staining, whereas the secretion granules of the serous cells were positive only over the halo (Fig. 3).

The mucus produced by the bovine submandibular gland also contains sialylated glycoproteins, many of them terminated by the Neu5Ac(α2,6)GalNAc disaccharide (Gottschalk & Graham, 1959). Indeed, on semithin sections of this gland we found intense staining of mucous cells and excretory duct epithelia, while serous cells were somewhat less intensely stained (Fig. 4).

When adjacent semithin sections from bovine submandibular gland were incubated with SNA followed by fetuin–gold complex, the same pattern of staining was observed as found with the SNA–g complex. However, the intensity of staining obtained (even with 250 μg ml^{-1} of SNA) over mucous and serous cells was much less, while striated ducts exhibited an intensity of staining comparable to that seen with the SNA–g complex (Fig. 5).

In thin sections from rat liver, gold particle label was detectable in two *trans* Golgi apparatus cisternae and throughout the *trans*-tubular network continuous with them (Fig. 6), supporting our previously reported data on Golgi apparatus distribution of immunolabel for α2,6 sialyltransferase and binding sites for the *Limax flavus* lectin (Roth *et al.*, 1985). In addition, secondary lysosomes, autophagosomes, endosome-resembling structures and the plasma membrane were labelled with SNA–g (Fig. 6). Nucleus, nuclear envelope, rough and smooth endoplasmic reticulum, mitochondria and peroxisomes were not labelled (Fig. 6). The same pattern of labelling was also found in the cultured hepatoma cells (Fig. 7).

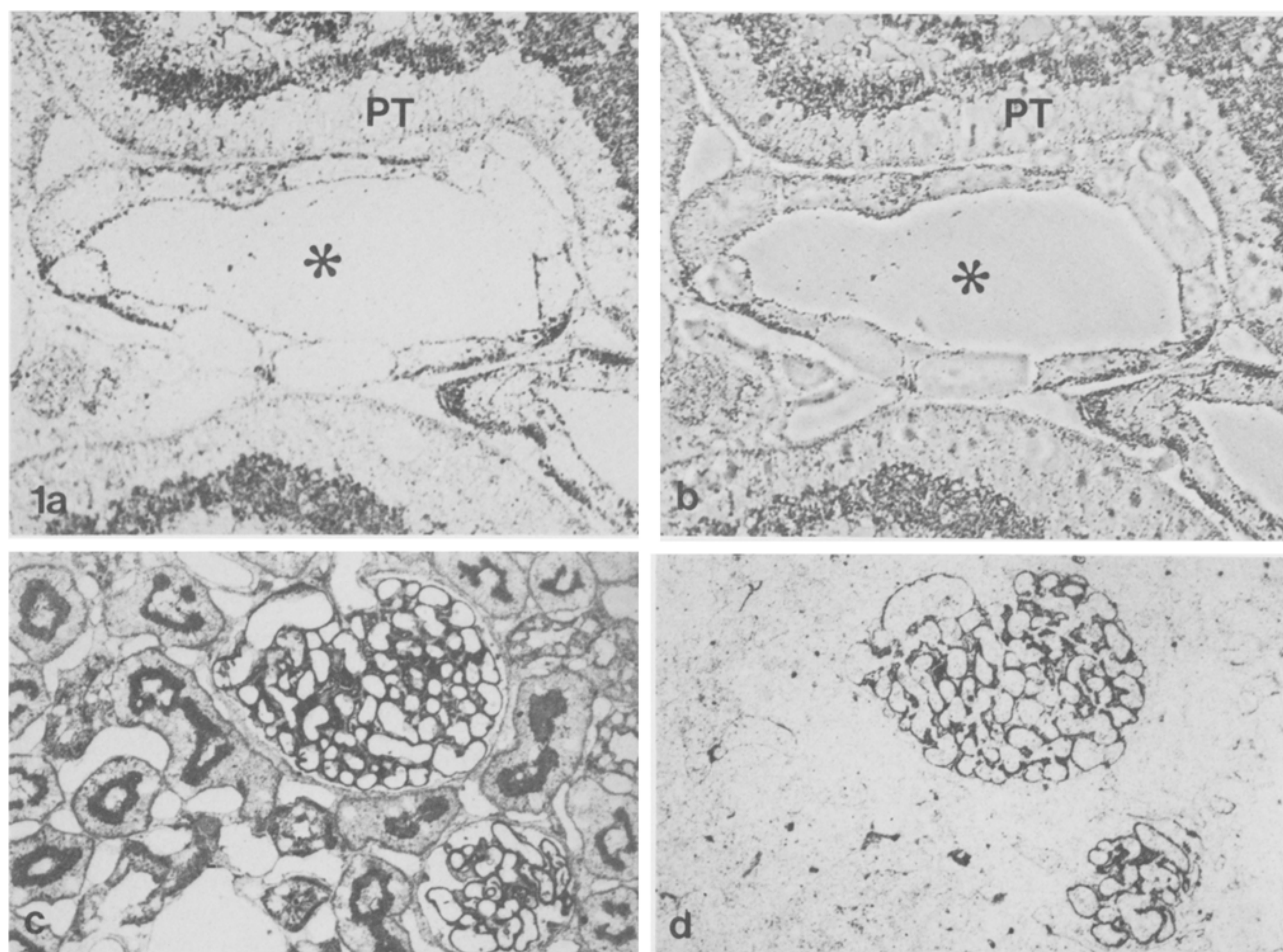

Fig. 1. Semithin Lowicryl K4M sections, rat kidney, SNA–g complex. In this and all subsequent light microscopical photographs, the gold particle signal was amplified by photochemical silver staining. (a,b) Intense reaction is seen over proximal tubular epithelia (PT). Positive and negative cells are found in the cortical portion of the thick ascending limb of Henlé (asterisk). (c,d) Consecutive serial semithin sections to demonstrate the differential effect of neuraminidase pretreatment. (c) SNA–g complex results in intense glomerular and tubular staining. (d) Pretreatment with neuraminidase abolishes tubular staining; glomerular staining is unaffected. (a,c,d) Bright field, (b) phase contrast. (a,b) × 500; (c,d) × 200.

We also prepared SNA–g complexes with 10-nm gold particles obtained with the tannic acid–citrate technique reported by Slot & Geuze (1985). Such complexes were applied for labelling after additional centrifugation on a 10–30% sucrose gradient. In contrast to SNA–g made with ascorbic acid gold, such complexes gave in addition to the specific labelling pattern high background over the nucleus and mitochondria (Fig. 8). Although similar background is obtained by us with protein A–gold prepared with tannic acid–citrate gold, it can be eliminated by the inclusion of 0.075% Triton X-100 and Tween 20 in the diluting buffer (Taatjes *et al.*, 1987b). However, the inclusion of detergent into the dilution buffer for SNA–g greatly reduced the staining. This finding was not altogether surprising since a distinct hydrophobic binding region has been detected adjacent to the carbohydrate binding site of SNA (Shibuya *et al.*, 1987a).

Cytochemical controls

Due to the abundance of the Neu5Ac(α2,6)Gal/GalNAc sequence in bovine submandibular gland we chose this tissue to perform a whole panel of cytochemical controls as discussed in detail below. Selected controls on other tissues are described where appropriate.

The staining over mucous and serous cells was abolished or greatly reduced when the SNA–g complex was preabsorbed with the following sugars or glycoproteins: 0.1 mM α2,6 sialyllactose, 5 mM α2,3 sialyllactose, 50 mM lactose, 100 mM GalNAc, 100 mM galactose, 100 μg ml^{-1} fetuin, and 100 μg ml^{-1} orosomucoid (Fig. 9). The following sugars or glycoproteins when used for preabsorption of the SNA–g complex yielded no influence on the staining: 100 mM glucose, 100 mM galactosamine, 100 mM *N*-acetylneuraminic acid, 100 μg ml^{-1} asialofetuin, and 100 μg ml^{-1} asialoorosomucoid.

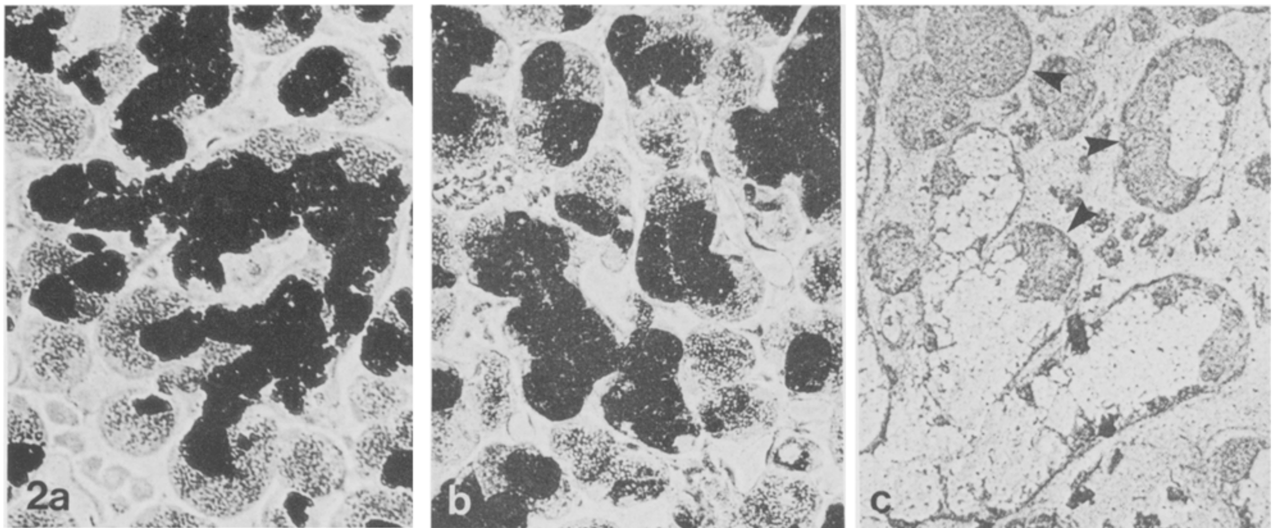

Fig. 2. Semithin Lowicryl K4M sections, sheep submandibular gland. Staining with SNA–g complex is equally intense over the submandibular gland with (a) or without (b) neuraminidase pretreatment of the sections. (c) Neuraminidase pretreatment was effective in sialic acid removal as evidenced by the lack of staining over the mucus droplets with the *Limax flavus* lectin. Some neuraminidase-resistant staining remains over the serous cells (arrowheads). (a–c) Phase contrast. × 125.

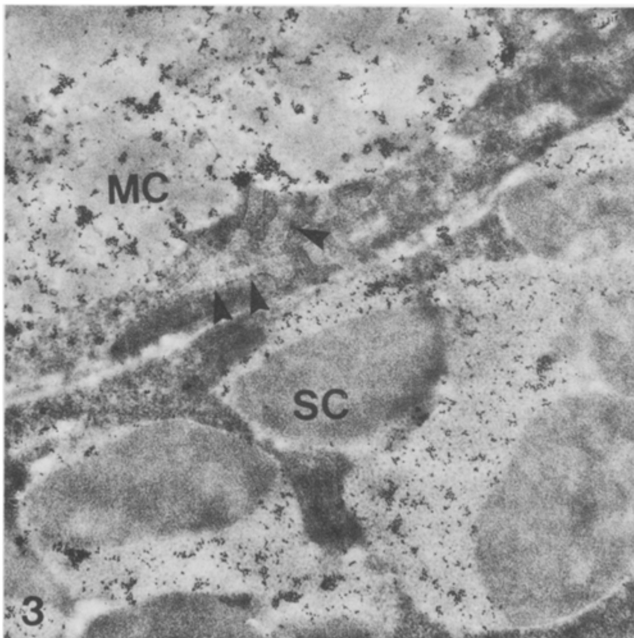

Fig. 3. Ultrathin Lowicryl K4M section from sheep submandibular gland showing binding of SNA–g complex to the mucus droplets of a mucous cell (MC) and the halo but not the core of secretion granules of serous cell (SC). Note absence of label over the rough endoplasmic reticulum of the mucous cell (arrowheads). × 23 000.

Semithin sections from bovine and sheep submandibular gland treated with neuraminidase before incubation with SNA–g complex exhibited the same, or even greater, intensity of staining as observed for sections exposed to acetate buffer alone or for sections with no pretreatment (Fig. 2). The effectiveness of the neuraminidase treatment was checked by exposing sections, in parallel to those above, to neuraminidase followed by staining for sialic acid with the *Limax flavus* lectin/fetuin–gold complex (Roth *et al.*, 1984). This treatment abolished all staining with this lectin (some neuraminidase-resistant sialic acid was still observed in the serous cells), demonstrating that sialic acid had been effectively removed (Fig. 2). Indeed, neuraminidase pretreatment of ultrathin sections form rat liver followed by SNA–g complex resulted in an absence of labelling (Fig. 6). Pretreatment of semithin sections from rat kidney with neuraminidase followed by SNA–g complex yielded abolition of staining over all structures except the glomeruli (Fig. 1). Similar sections treated with neuraminidase followed by incubation with *Helix pomatia* lectin–gold showed a very intense and broad staining which contrasts with the highly discrete glomerular staining obtained with this lectin on non-neuraminidase-treated sections (Roth *et al.*, 1983). Thus, we considered that the staining observed on neuraminidase-pretreated sections from submandibular gland and kidney was due to binding of the SNA–g complex to the penultimate GalNAc residue revealed by the enzymic digestion.

Spot blot analysis

In order to test whether the Neu5Ac(α2,6)Gal/GalNAc sequence in glycoproteins is also recognized by the SNA–g complex after immobilization onto nitrocellulose, spot blots were performed. As shown in Fig. 10, a positive reaction was observed with spot-blotted fetuin and asialofetuin, whereas no staining was detectable for BSA. Apparently, the positive reaction with asialofetuin is due to exposure of penultimate GalNAc residues, as discussed above, since the *Limax flavus* lectin stained only the fetuin (Fig. 10).

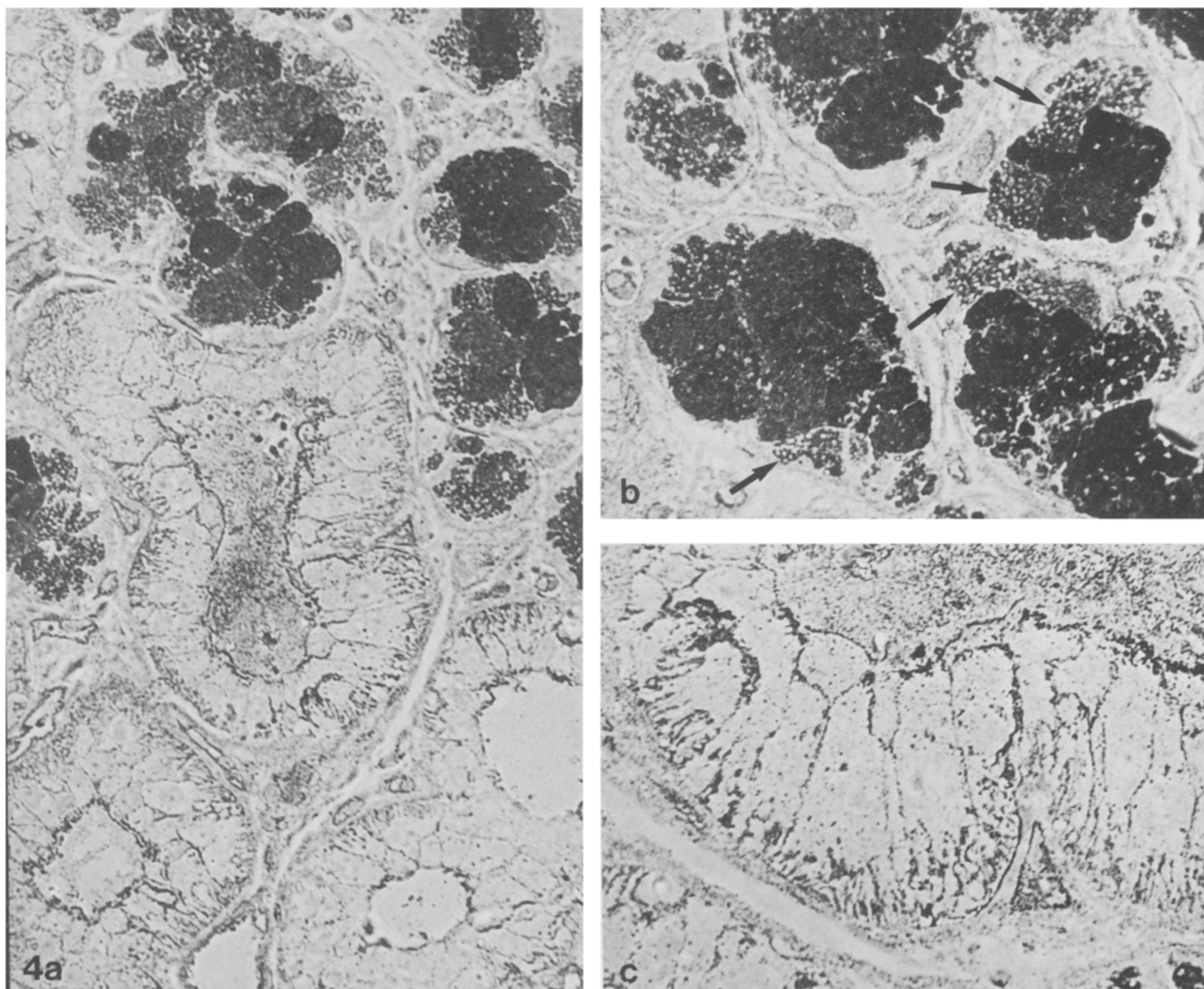

Fig. 4. Semithin sections from bovine submandibular gland, phase contrast imaging. The SNA–g complex stains the mucus cells more intensely than the serous cells (a,b). At higher magnification the unstained secretion granule core of serous cells becomes evident (arrows in (b)). (a) × 242; (b) × 310; (c) × 533.

Lectin overlay analysis

The SNA–g complex stained several bands but this interaction could not be inhibited in the control incubations.

Discussion

In the first report detailing its isolation and purification, SNA was characterized to be a lactose-specific lectin since low concentrations of lactose were inhibitory in haemagglutination tests; however, GalNAc was an equally potent inhibitor and, to a slightly lesser extent, Gal (Broekaert *et al.*, 1984). Subsequently, more extensive characterization of the carbohydrate-binding properties of SNA led to the realization that it binds to highly sialylated glycoproteins (Shibuya *et al.*, 1987a). Specifically, a marked preference for the Neu5Ac(α2,6)Gal/GalNAc disaccharide unit was found, with an affinity 20–150 times greater than for the Neu5Ac (α2,3)Gal/GalNAc sequence. Advantage was taken of this discriminative property to separate on a SNA-Sepharose column 2,6-linked from 2,3-linked isomers and for the fractionation of oligosaccharides and glycopeptides based on their number of Neu5Ac(α2,6)Gal units (Shibuya *et al.*, 1987b). In their earlier study, Shibuya *et al.* (1987a) found that Gal, GalNAc, and various oligosaccharides containing these sugar residues at the non-reducing termini were inhibitory towards SNA in hapten inhibition studies in the range of 10–30 mM. Moreover, the introduction of the nonpolar *p*-nitrophenol aglycone into Gal or GalNAc enhanced their inhibitory power to equal that of the Neu5Ac(α2,3)Gal sequence. Based on these observations, a tentative model of the carbohydrate

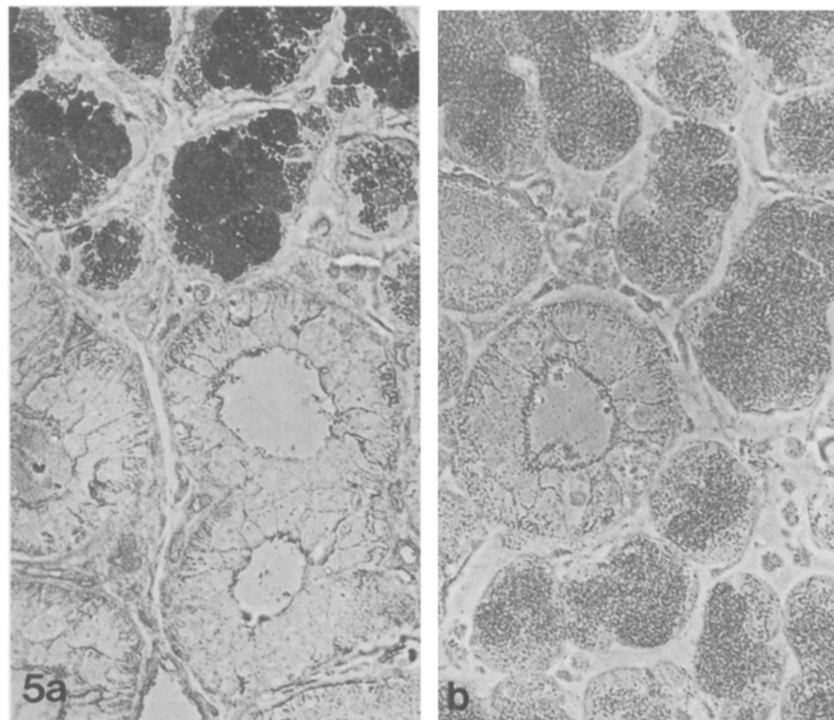

Fig. 5. Comparison of the staining obtained on bovine submandibular gland sections with the SNA–g complex (a) and the SNA/fetuin–gold complex (b). (a,b) Phase contrast; × 320.

binding site of SNA was proposed: two contiguous carbohydrate binding subsites to accommodate Neu5Ac and Gal/GalNac, respectively, and a hydrophobic binding region adjacent to the Gal/GalNAc subsite.

In the present study, we used the SNA in a direct lectin–gold technique and in a cytochemical affinity technique in conjunction with fetuin–gold complexes. On semithin sections from the bovine submandibular gland, the SNA–g complex yielded very intense staining, whereas the SNA followed by fetuin–gold complexes resulted in a much weaker staining. The latter result is apparently due to the bivalency of SNA (Shibuya *et al.*, 1987a) leading in many cases to both sugar combining sites interacting with tissue glycoconjugates and thus preventing the visualization of some lectin molecules by the fetuin–gold complex. Although we have tested SNA–g complexes only on sections of Lowicryl K4M-embedded tissues, we anticipate that the technique would work as well on sections from paraffin, LR resins and Epon-embedded tissues as demonstrated previously for other lectins [for review, see Roth (1987) and Ellinger & Pavelka (1985)].

Our interest in applying the SNA stems from our investigations on immunolocalization of the α2,6-sialyltransferase (Roth *et al.*, 1985, 1986). In these studies we had used the *Limax flavus* lectin to identify the product of the sialyltransferase activity. From the abovementioned data on the sugar specificity of SNA, it appeared that this would be the most appropriate lectin for the localization of the α2,6-sialyltransferase product. Indeed, we found in rat liver the Golgi apparatus distribution of SNA-binding sites to be coincident with immunolabel for α2,6-sialyltransferase [this study, and Roth *et al.* (1985)]. Most importantly, the SNA–g staining on ultrathin sections could be completely abolished by pretreatment of the sections with neuraminidase, confirming the requirement of sialic acid for high-affinity binding of the lectin. However, when a whole battery of controls was performed on semithin sections from bovine submandibular gland the results were less clear. Although preabsorption of the SNA–g complex with a variety of sugars and glycoproteins basically confirmed the data from quantitative precipitation and hapten inhibition (Shibuya *et al.*, 1987a), certain limitations with respect to the relative inhibitory power of sialyllactose as compared to lactose, Gal or GalNAc were observed. Clearly, α2,6-sialyllactose was the most potent inhibitor of the SNA–g staining, requiring concentrations only in the micromolar range for full inhibition. However, in a similar manner, as much as 50 mM of lactose and 100 mM of both Gal and GalNAc were required for complete inhibition of the cytochemical staining. Most surprising was the observation that pretreatment of the sections with neuraminidase had no effect on the intensity of SNA–g staining. As judged by the absence of staining with the *Limax flavus* lectin performed in parallel on adjacent sections, the

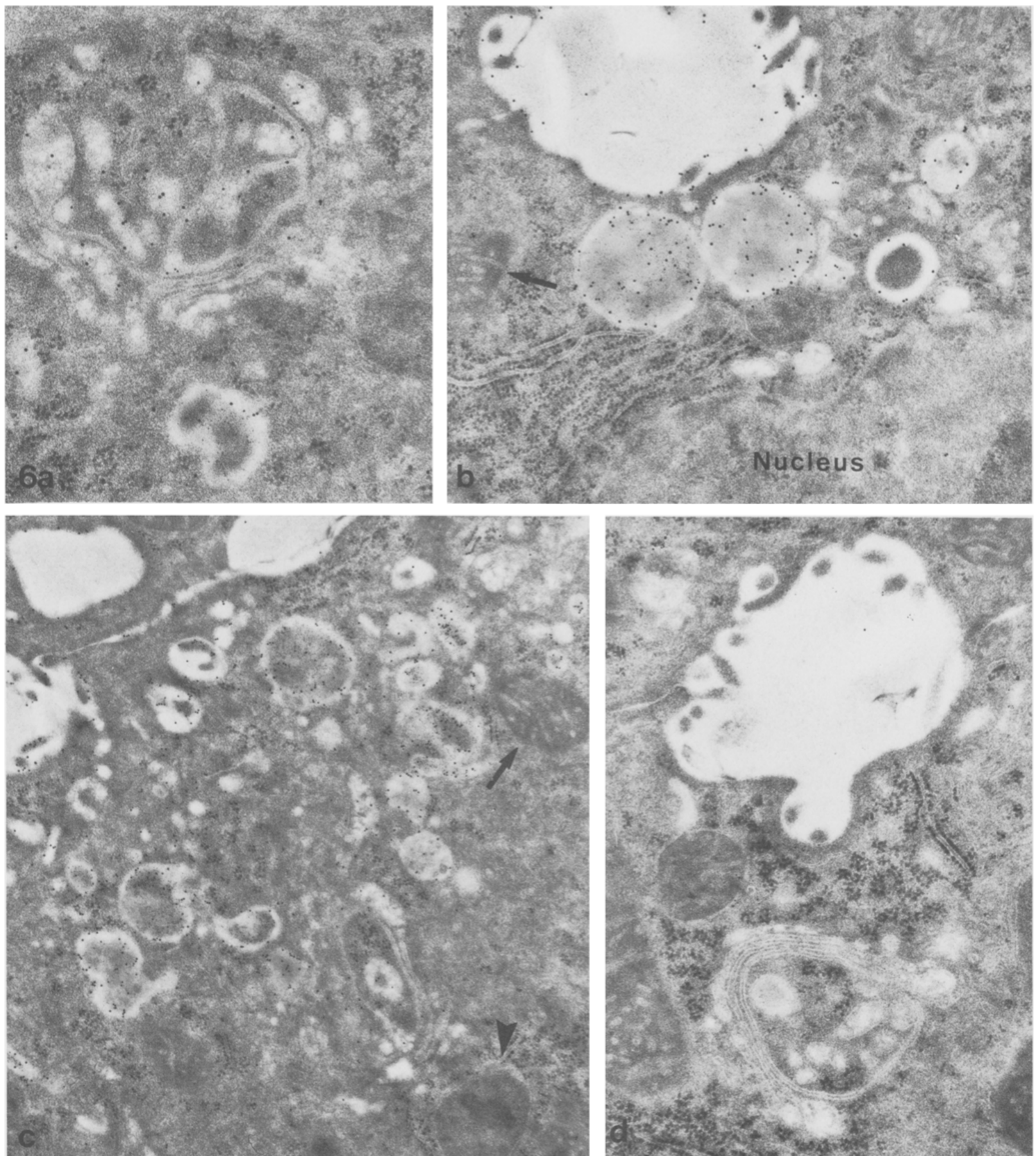

Fig. 6. Ultrathin sections from rat liver incubated with SNA–g complex exhibit gold particles over *trans* cisternae and *trans*-tubular network of the Golgi apparatus, various lysosomal and endosomal structures as well as the plasma membrane (a–c). Pretreatment of the sections with neuraminidase followed by SNA–g complex results in absence of staining (d). Arrows, mitochondria; arrowhead, peroxisomes. (a) × 46 300; (b) × 33 700; (c) × 28 000; (d) × 37 000.

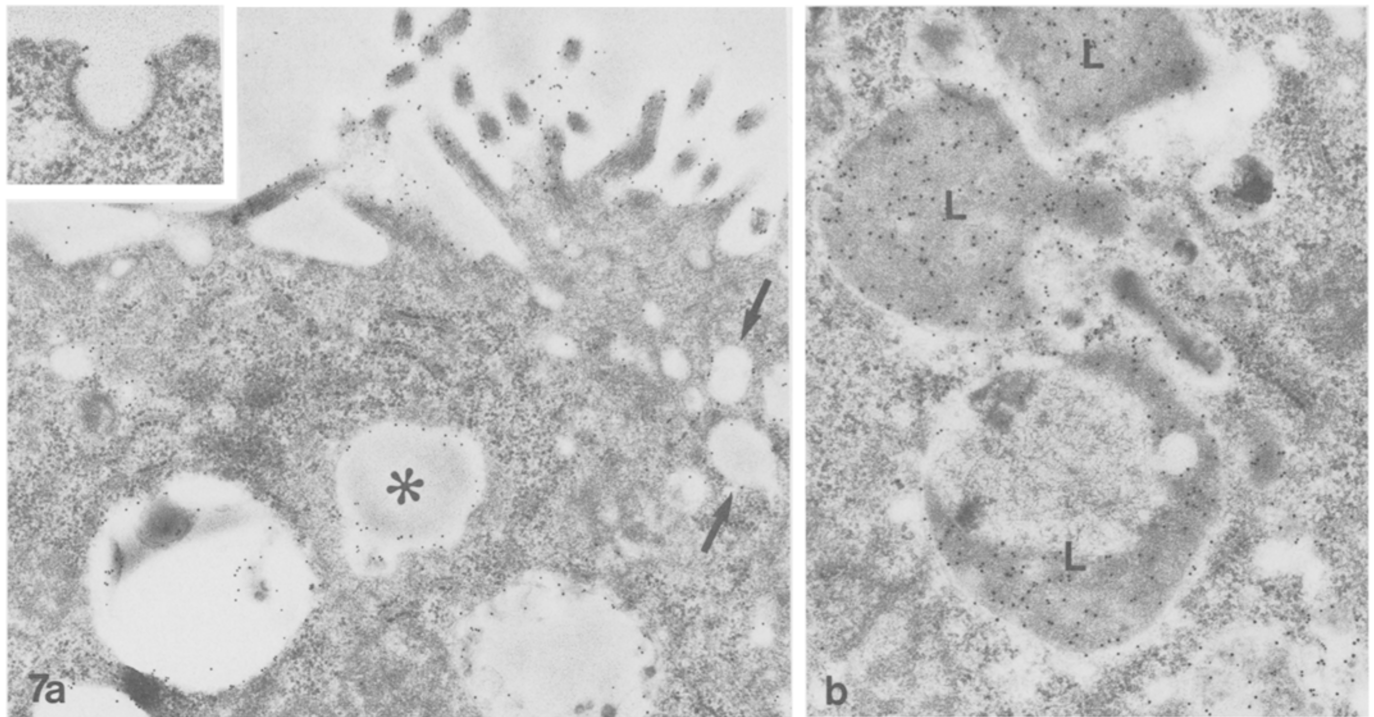

Fig. 7. Ultrathin sections from cultured rat hepatoma cells. SNA–g complex stains microvilli (a) and coated pits (inset in (a)) of the plasma membrane as well as small cytoplasmic vesicles (arrows) and lysosome-like bodies (L). Note that some lysosomes are labelled only at the inner surface of the limiting membrane (asterisk in (a)) whereas others exhibit also label over the content (b). (a) × 27 500; (b) × 37 000; inset × 46 000.

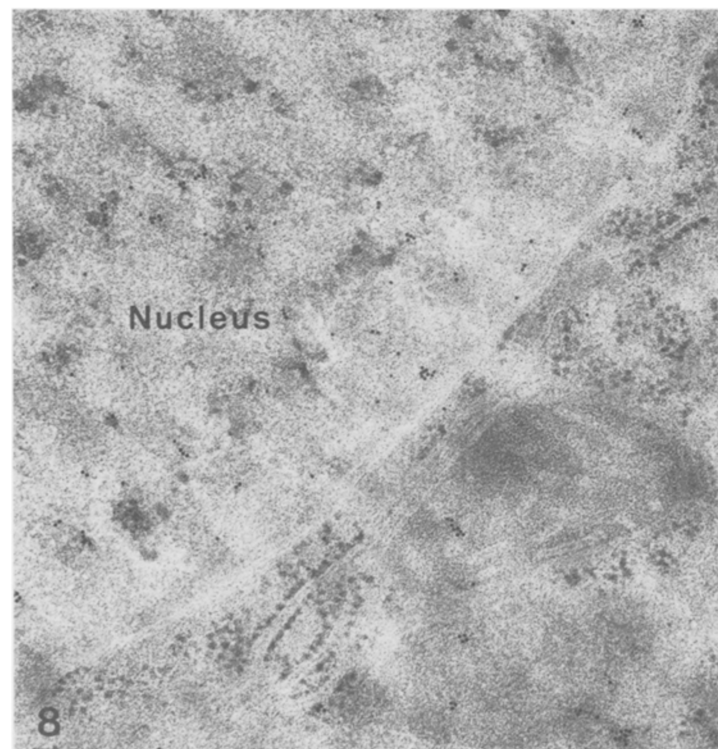

Fig. 8. Ultrathin section from rat liver. SNA–g complex prepared with tannic acid/citrate gold binds non-specifically to the nucleoplasm and mitochondria. × 62 000.

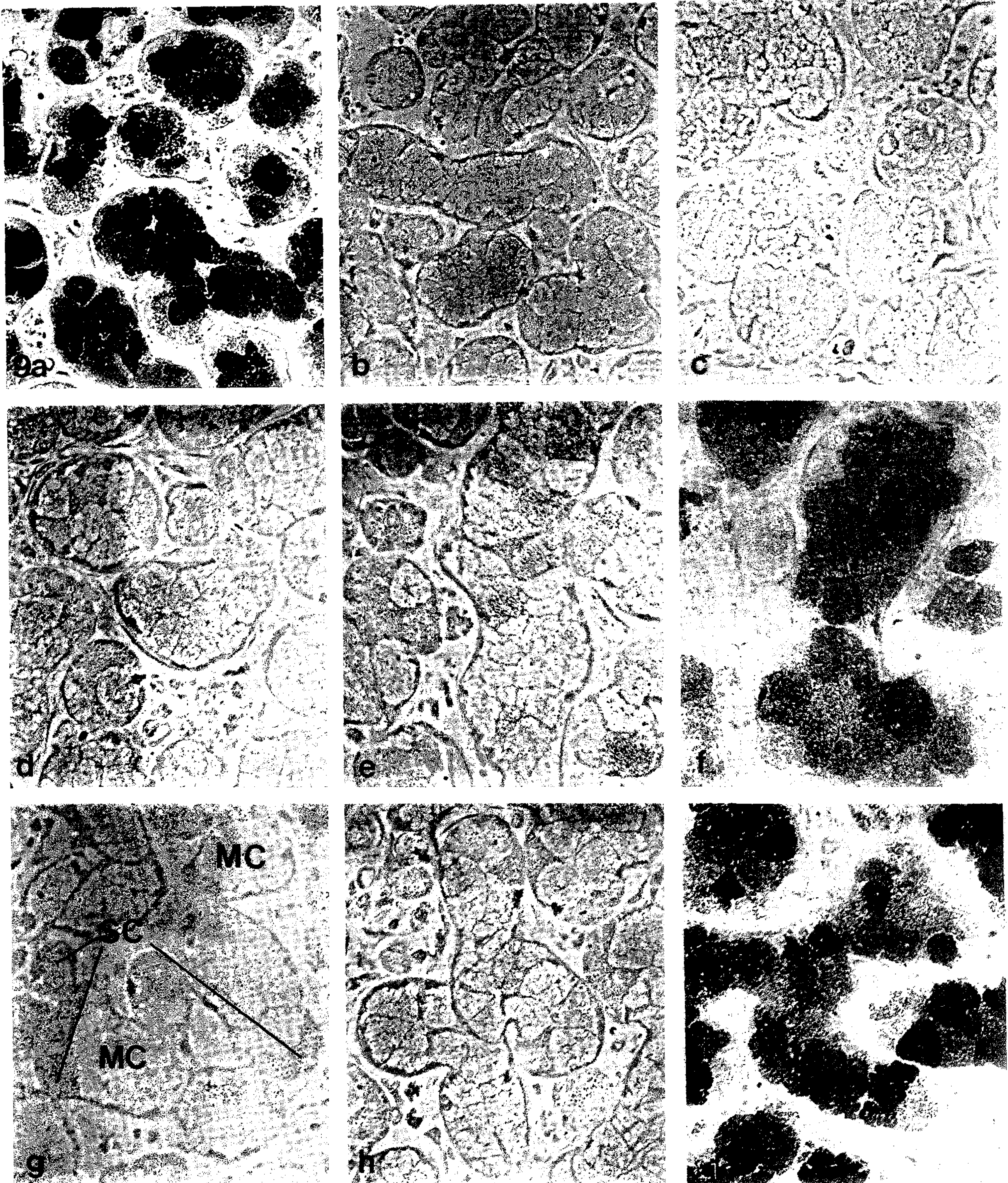

Fig. 9. Influence of various saccharides and glycoproteins on the SNA–g complex staining on semithin sections from bovine submandibular gland. (a) SNA–g complex. The staining becomes negative when the SNA–g was preincubated with 0.1 mM α2,6 sialyllactose (b), 100 mM GalNAc (c), 50 mM lactose (d), or 100 μg orosomucoid (e). Addition of 0.1 mM α2,3 sialyllactose (f) decreased the intensity of staining whereas 5 mM α2,3 sialyllactose (g) inhibited staining over the mucous cells (MC) and greatly reduced it over the serous cells (SC). Addition of 100 μg fetuin abolished staining over mucous cells but serous cells showed residual staining (h); 100 μg asialofetuin had no influence on the staining result (i). (a)–(i) Phase contrast. × 200.

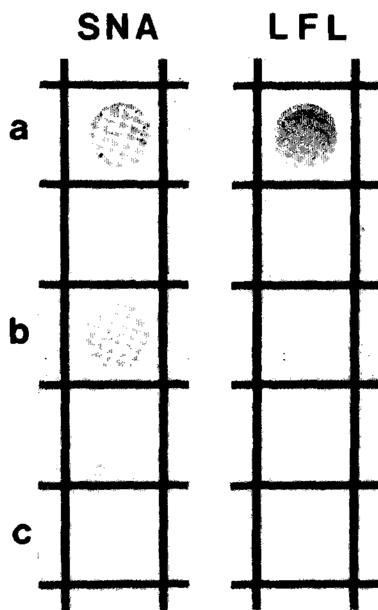

Fig. 10. Dot blot analysis of the binding of SNA–g complex and the *Limax flavus* lectin to nitrocellulose immobilized glycoproteins. SNA–g binds to both fetuin and asialofetuin, whereas the *Limax flavus* lectin binds only to fetuin. (a) Fetuin; (b) asialofetuin; (c) bovine serum albumin. LFL, *Limax flavus* lectin/fetuin–gold.

enzymic removal of sialic acid had been effective. Moreover, treatment of rat kidney sections with neuraminidase followed by SNA–g complex resulted in an abolition of all staining over tubular elements, but had no effect on the staining of glomeruli. A similar reactivity of the SNA–g complex was also observed on nitrocellulose immobilized fetuin and asialofetuin. Although the *Limax flavus* lectin reacted only with fetuin, the SNA–g reacted with both fetuin and asialofetuin. These data underscore the necessity of performing cytochemical controls with a spectrum of sugars and glycoproteins in addition to the expected inhibitory substrates. As evident from our present results, the use of glycosidases to remove the substrate for lectin binding to tissue sections is of utmost importance. The enzymic removal of sialic acid from bovine submandibular gland and rat kidney sections revealed that the SNA–g complex also has a high affinity for a high density of terminal GalNAc residues. This finding is in contrast to observations made on purified glycoproteins (Shibuya *et al.*, 1987a). The structural diversity of oligosaccharide chains present in the various tissues and cells may thus hinder the applicability of the SNA–g complex such that in the practical terms of cytochemical staining the SNA–g complex may not always distinguish between oligosaccharide chains terminated by the Neu5Ac(α2,6) GalNAc sequence or by GalNAc alone, especially if the latter sugar is abundantly present. Hence the importance of conducting enzymic and sugar inhibition controls.

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