Histochemistry (1989) 92:515–522

Histochemistry

Light and electron microscopic detection of (3 Gal β1,4 GlcNAc β1) sequences in asparagine-linked oligosaccharides with the *Datura stramonium* lectin*

G. Egea[1], I.J. Goldstein[2], and J. Roth[1]**

[1] Interdepartmental Electron Microscopy, Biocenter, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

[2] Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109-0606, USA

Accepted June 30, 1989

Summary. The *Datura stramonium* lectin recognizes with high affinity the disaccharide *N*-acetyllactosamine (Gal β1,4 GlcNAc). We have developed a highly specific cytochemical affinity technique in which an ovomucoid-gold complex serves as second step reagent for the visualization of this lectin bound to reactive sequences present in tissue sections. The lectin binding sites were detected in semithin and ultrathin sections of aldehyde-fixed and low temperature Lowicryl K4M embedded tissues. For light microscopical labeling the photochemical silver reaction for signal amplification was required. The application of this technique for the detection of *N*-acetyllactosamine containing asparagine-linked oligosaccharides in various intracellular organelles and the plasma membrane is demonstrated.

Introduction

The lectin from *Datura stramonium* recognizes with high affinity *N*-acetyllactosamine and its repeating disaccharide sequences (3 Gal β1,4 GlcNAc β1) or an outer mannose residue substituted at C-2,4, and C-2,6 by *N*-acetyllactosamine of asparagine-linked tri- and tetrabranched oligosaccharides (Crowley et al. 1984; Cummings and Kornfeld 1984; Green et al. 1987a, b; Yamashita et al. 1987). The lectin binding is not influenced by the presence of a bisecting *N*-acetylglucosamine residue nor by the inner core portion of the oligosaccharide. However, binding is abolished if substitution by terminal α2,6 or α2,3-linked *N*-acetylneuraminic acid or an α-L-fucosyl group has occurred. In order to localize these commonly occurring disaccharide sequences we have investigated the *Datura stramonium* lectin using a cytochemical affinity technique. Among various glycoprotein-gold complexes prepared, the ovomucoid-gold complex proved to be the most suitable second-step reagent. This cytochemical affinity technique permitted the postembedding localization of intra- and extracellular lectin binding sites in various tissues embedded at low temperature in Lowicryl K4M. Identical conditions of incubation could be used for both light and electron miroscopic localization studies. For light microscopy, signal amplification with the photochemical silver reaction was required. The specificity of lectin binding to the tissue sections was established by various sugar and glycoprotein inhibition experiments, as well as enzymatic and chemical oligosaccharide modification reactions performed on the tissue sections. We propose the *Datura stramonium* lectin as a useful cytochemical reagent for the detection of the *N*-acetyllactosamine sequence.

Materials and methods

Reagents. Datura stramonium lectin was affinity-purified from *Datura stramonium* seeds as previously reported (Crowley and Goldstein 1981), or affinity-purified lectin was purchased from Sigma Chemical Co, Ltd. (St. Louis, MI) as were neuraminidase (from *Clostridium perfringens*), β-galactosidase (from *Diplococcus pneumoniae*), fetuin, ovomucoid, *N*-acetylglucosamine, *N*-acetylgalactosamine, *N*-acetylneuraminic acid, D-galactose, D-glucosamine and D-galactosamine. Tetrachloroauric acid, L-fucose, D-glucose, lactose, polyethylene glycol (m. wt. 20000), sodium nitrite and paraformaldehyde were from Merck (Darmstadt, FRG); glutaraldehyde (purissimum) and polyvinyl pyrolidone (m. wt. 40000) from Fluka (Buchs, Switzerland). Endo-β-*N*-acetylglucosaminidase (endo H; from *Streptomyces griseus*) was from Boehringer (Mannheim, FRG), *N*-glycanase (from *Flavobacterium meningosepticum*) from Genzyme (Boston, MA), and *N*-acetyllactosamine from Bio-Carb Chemicals (Lund, Sweden). Asialoorosomucoid was a generous gift from Dr. J.C. Paulson (UCLA). Asialofetuin, β-galactosidase (from bovine testes), and *N,N'*-diacetylchitobiose were avail-

Table 1. Dilutions of glycoprotein-gold complexes for incubation of semithin and ultrathin sections of Lowicryl K4M embedded tissues

Glycoprotein	Gold particle size[a] [nm]	Dilution [$O.D._{525nm}$]
Ovomucoid	5	0.05
Ovomucoid	8	0.20
Ovomucoid	15	0.20
Fetuin	10	0.20
Asialofetuin	15	0.15
Asialoorosomucoid	15	0.20

[a] Dilutions were made with PBS containing 0.2% polyethylene glycol or Carbowax 20 *M* (15-nm gold) or 1% bovine serum albumin, 0.05% Triton X-100 and 0.05% Tween 20 (5-nm, 8-nm and 10-nm gold)

* This study was supported by the Swiss National Science Foundation grant nr. 31-26273.89 (to J.R.) and GM 29470 from the National Institutes of Health (to I.J.G.). Dr. G. Egea was a recipient of a European Molecular Biology Organization long term fellowship.

** To whom offprint requests should be sent

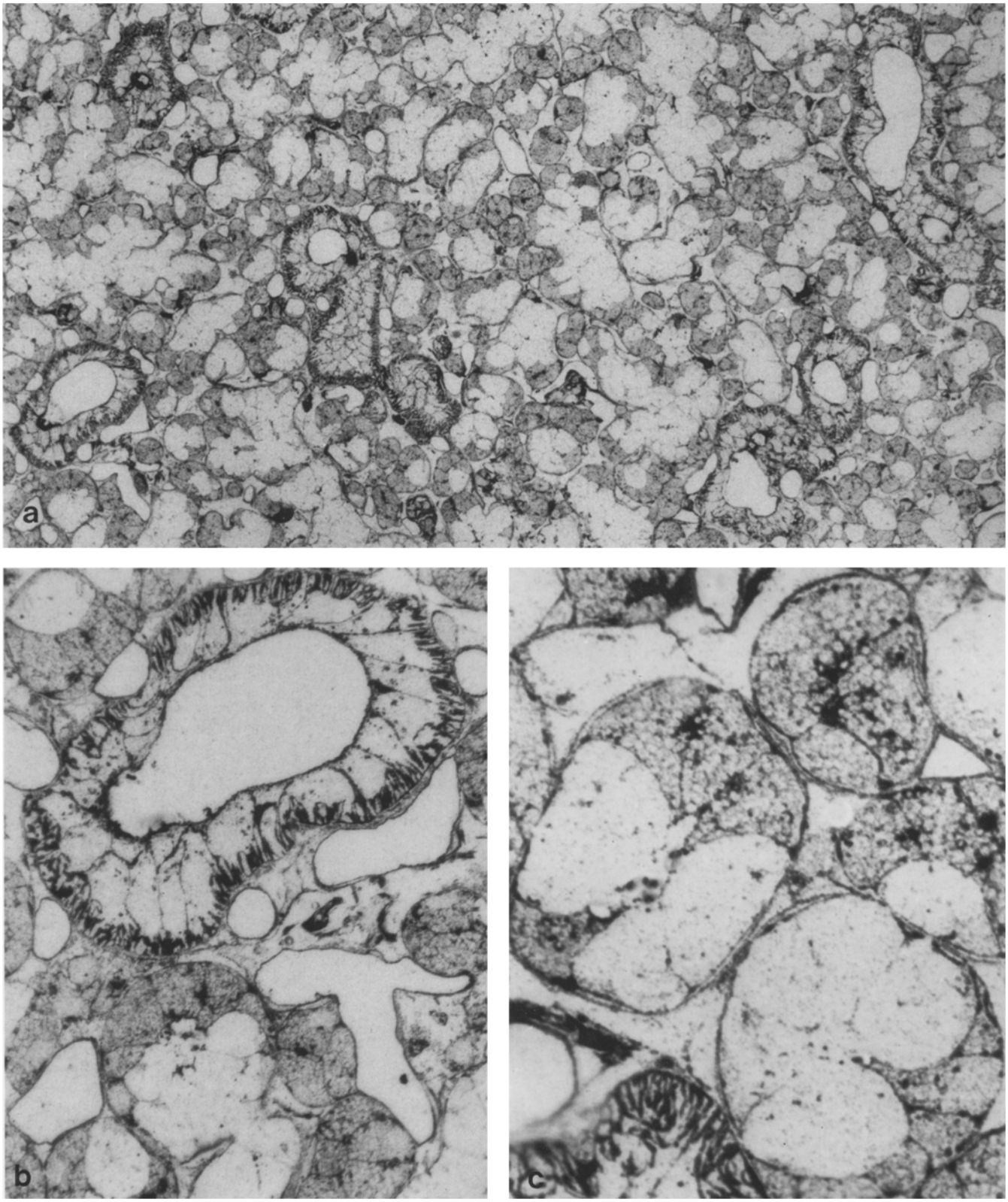


Fig. 1 a–c. Sheep submandibulary gland, semithin Lowicryl K4M sections. Incubation with the *Datura stramonium* lectin followed by ovomucoid-gold (8 nm) complexes. All positive structures appear black due to photochemical silver reaction; no counterstaining. The lectin staining of striated ducts and demilunes formed by the serous cells can be clearly appreciated at low magnification **a**. The staining of the plasma membrane of striated duct epithelia and some intracellular structures together with positive capillaries and demilunes is shown at higher magnification in **b**. A detail of mixed acini showing lectin staining at the cell surface of both serous and mucous cells (C). The content of the secretion granule of both cell types appears not stained but the serous cell granule periphery displays positivity (see Fig. 5 for electron microscopy). ×240 (**a**); ×750 (**b**); × 1150 (**c**)

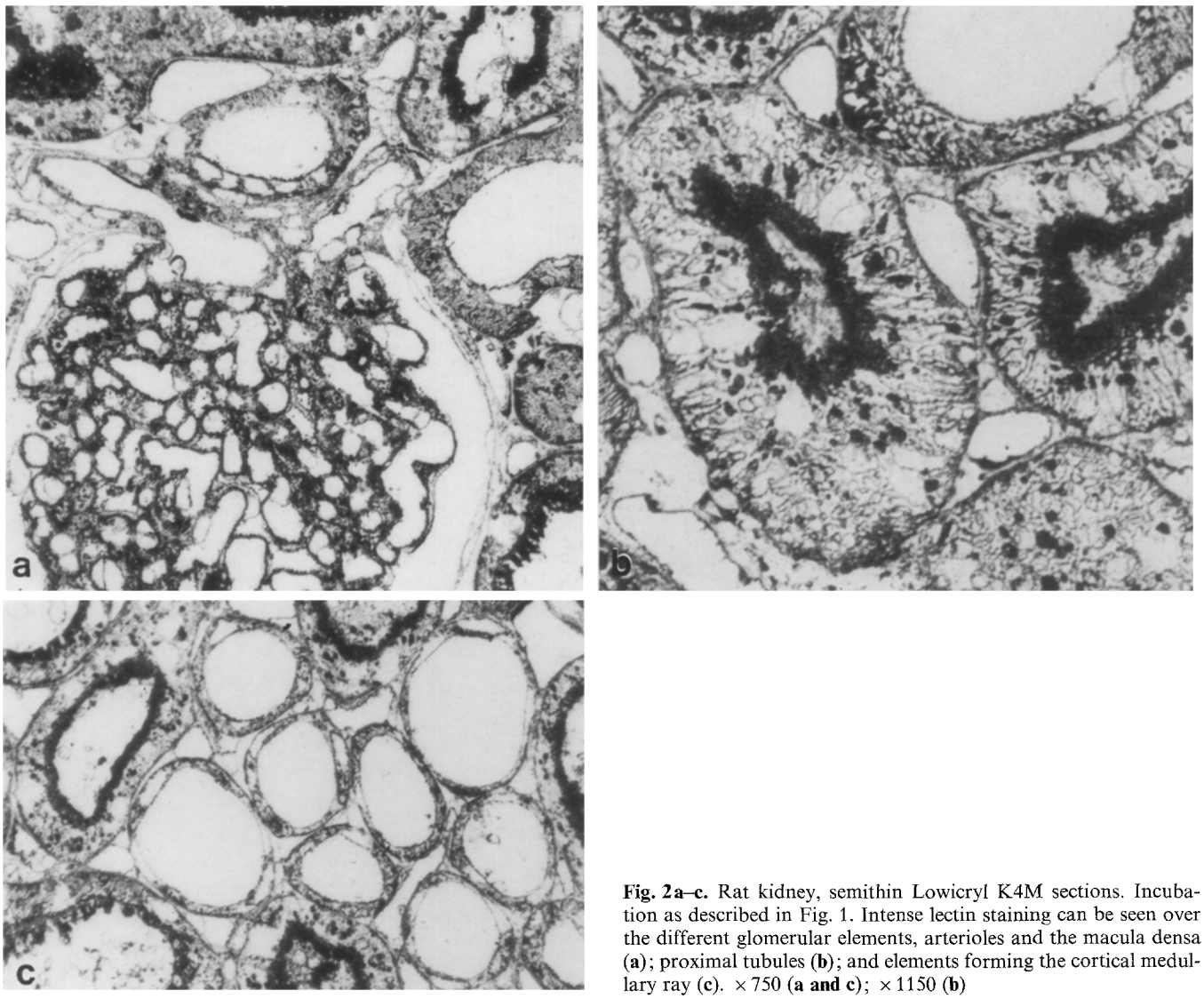
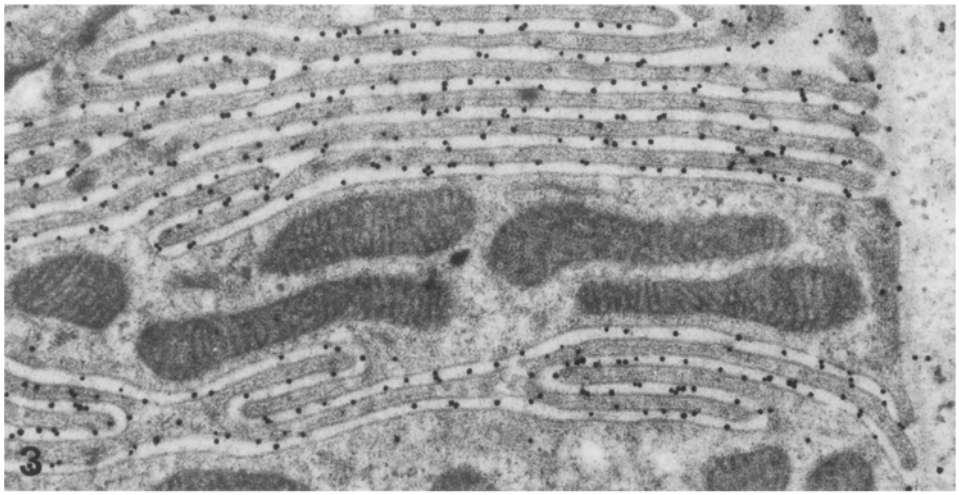


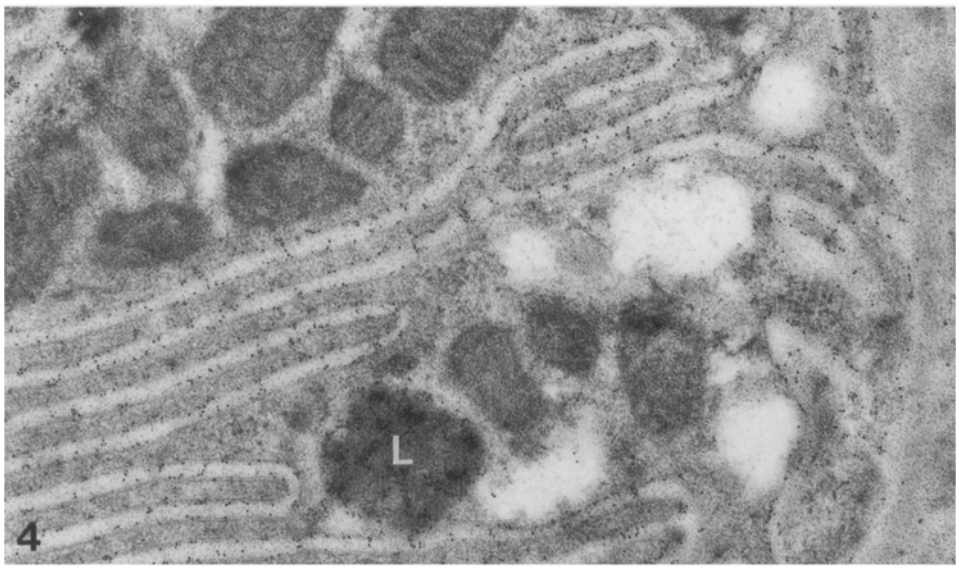
Fig. 2a–c. Rat kidney, semithin Lowicryl K4M sections. Incubation as described in Fig. 1. Intense lectin staining can be seen over the different glomerular elements, arterioles and the macula densa (**a**); proximal tubules (**b**); and elements forming the cortical medullary ray (**c**). ×750 (**a and c**); ×1150 (**b**)

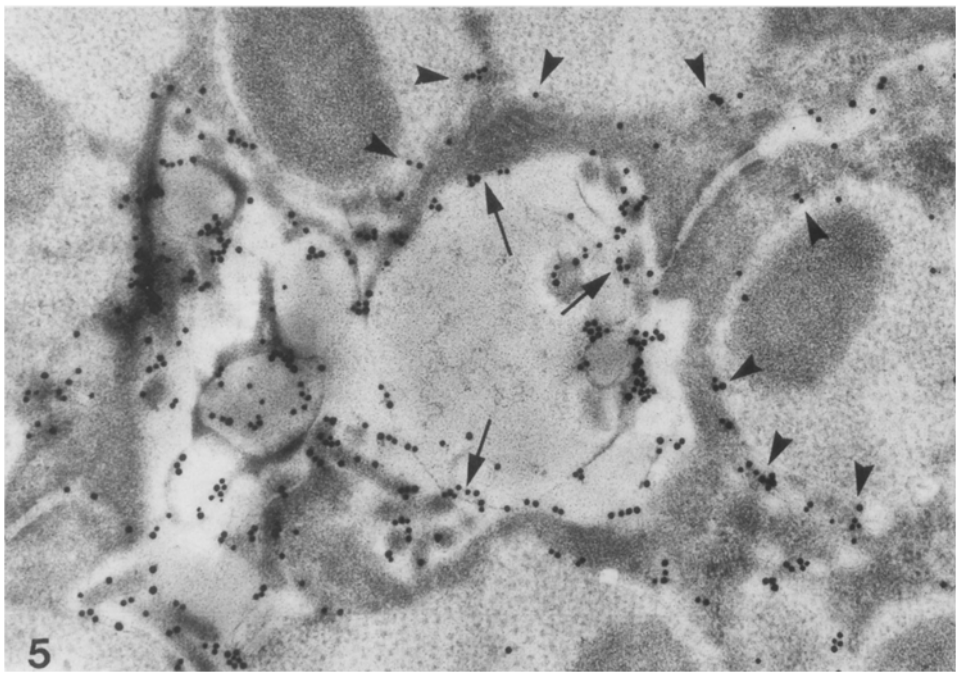
able from previous experiments in the laboratory of Dr. I.J. Goldstein (Ann Arbor).

Tissue processing. Rat duodenum and colon as well as bovine submandibular gland were fixed by immersion in 3% formaldehyde (freshly prepared from paraformaldehyde) −0.1% glutaraldehyde in PBS (0.15 *M* NaCl, 10 m*M* phosphate buffer, pH 7.4) for 2 h. Rat kidney, liver and parotid gland were fixed by vascular perfusion with the above mentioned fixative under conditions previously detailed (Roth et al. 1985). Sheep submandibular gland was perfused through the carotid artery first with PBS for 2 min followed by the above mentioned fixative for 10 min and immersion in the fixative for 1 h and 50 min. After fixation all tissues were washed with PBS three times for 10 min each and then treated with 50 m*M* NH_4Cl in PBS for 30 to 60 min to amidinate free aldehyde groups. Small tissue fragments were dehydrated at progressively lower temperatures to −35° C and embedded in Lowicryl K4M at −35° to −40° C (Carlemalm et al. 1982; Roth et al. 1981). Semithin sections (1 μm) were cut and mounted on poly-L-lysine-activated glass slides (Lucocq and Roth 1984). Ultrathin sections were placed on Parlodion/carbon coated nickel grids.

Preparation of glycoprotein-gold complexes. Monodisperse colloidal gold with a mean particle diameter of 15 nm was prepared according to Frens (1973). Gold particles of approximately 5, 8 and 10 nm in diameter were prepared according to Slot and Geuze (1985). The ovomucoid-gold complex using 15-nm gold particles was prepared as previously described (Roth 1983a). Ovomucoid was also complexed to 5-nm and 8-nm gold particles. For this purpose, the pH of the colloidal gold was adjusted to 4.8 and the optimal stabilizing amount of ovomucoid was estimated by the salt flocculation test (Roth and Binder 1978). To stabilize 10 ml of 5-nm or 8-nm gold sol, 250 μg ovomucoid and 150 μg ovomucoid, respectively, were required. For the preparation of asialofetuin-gold and fetuin-gold complexes the pH of the colloidal gold was adjusted to 7.4 and 5.4, respectively. For the stabilization of 10 ml colloidal gold (15 nm) 250 μg asialofetuin were needed; for 10 ml colloidal gold (10 nm) 200 μg fetuin were sufficient. For the preparation of asialoorosomucoid-gold complexes the pH of colloidal gold (15 nm) was adjusted to 5.0 and 65 μg of the glycoprotein were estimated to be the optimal stabilizing amount. The preparation of the glycoprotein-gold complexes was performed according to standard protocols (Roth 1983b). After complex formation, 1 ml of 1% polyethylene glycol or Carbowax 20 *M* was added to 25 ml crude complex. Purification and concentration of the crude complexes was achieved by ultracentrifugation for 45 min at $60000 \times g$ (15-nm gold) or $105000 \times g$ (5-nm, 8-nm and 10-nm gold). After centrifugation, the supernatant solution was removed as completely as possible and the sedimented glycoprotein-gold complexes resuspended in PBS containing 0.2 mg/ml of polyethylene glycol or Car-







Figs. 3–5. Sheep mandibulary gland, ultrathin Lowicryl K4M sections, *Datura stramonium* lectin/ovomucoid-gold complex staining. Details from the basal portion of striated duct epithelia with gold particle label (15 nm, **Fig. 3**; 5 nm, **Fig. 4**) along the outer surface of the plasma membrane and over a lysosome (*L*). Apical region of a serous cell (**Fig. 5**) with gold particle label along the inner aspect of the secretory granule limiting membrane (*arrowheads*) and the acinar plasma membrane (*arrows*). ×28000 (**Fig. 3**); ×35000 (**Fig. 4**); ×40000 (**Fig. 5**)

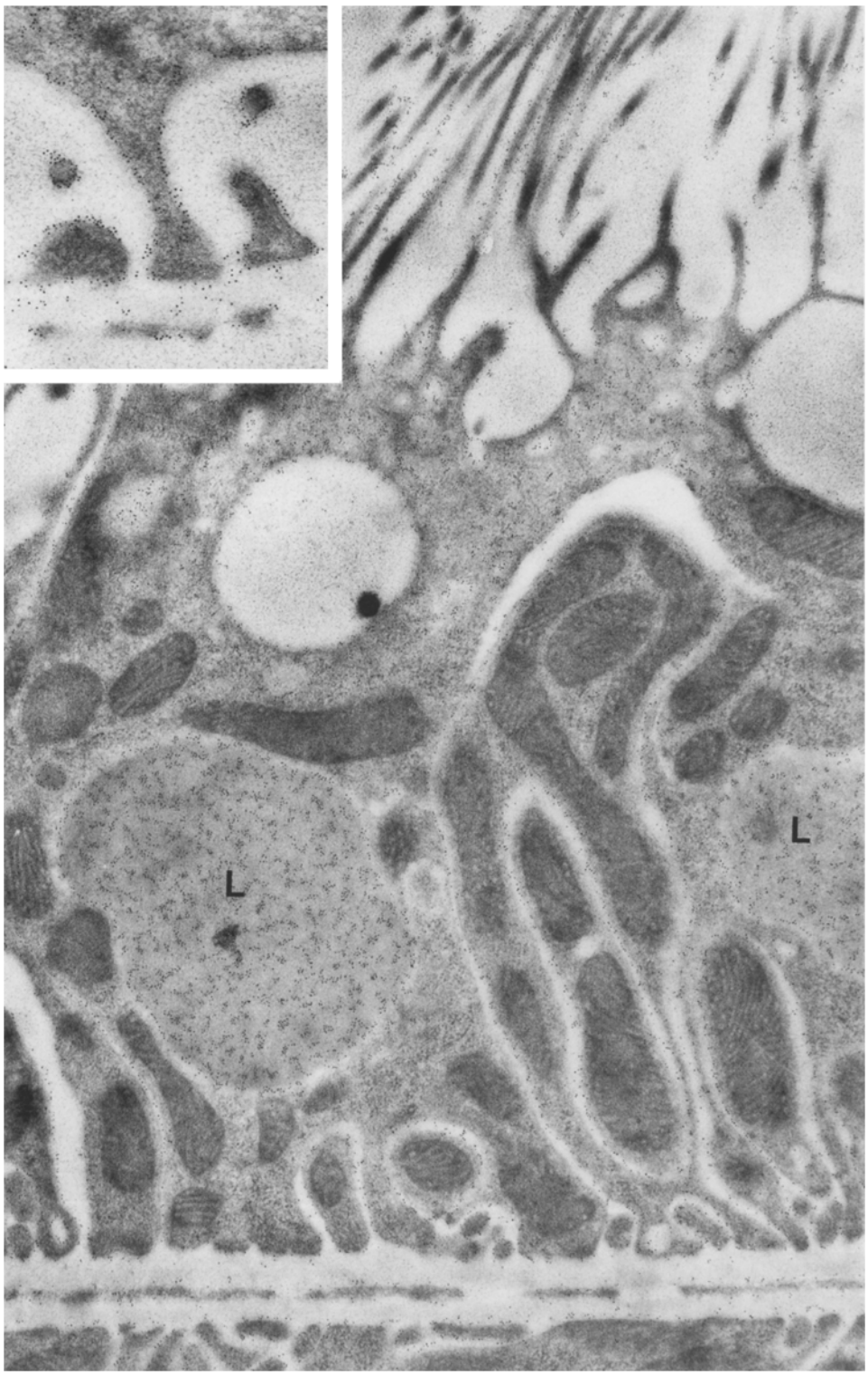


Fig. 6. Rat kidney, ultrathin Lowicryl K4M sections. In proximal tubular epithelia, lectin binding sites as indicated by the presence of gold particles (8-nm ovomucoid-gold complexes) are present along the apical (brush border) and basolateral plasma membrane, cytoplasmic vesicles and lysosomes (*L*). Glomerular podocyte and endothelial plasma membrane as well as basement membrane exhibit lectin labeling (*inset*). ×17000; ×43000 (*inset*)

bowax 20 *M*. Preparations made with 15-nm gold particles were stored at 4° C until use. The glycoprotein-gold complexes made with 5-nm, 8-nm and 10-nm gold were centrifuged into a 10%–30% glycerol/PBS gradient (Slot and Geuze 1982) to remove free tannic acid and aggregates.

Cytochemical labeling protocols. For light microscopical studies, semithin sections were covered with PBS for 5 min. Subsequently, the excess PBS was drained and the sections were incubated with *Datura stramonium* lectin (75–100 μg/ml in PBS) for 45 min at room temperature. Following two rinses with PBS for 5 min each the sections were incubated with one of the glycoprotein gold complexes for 45 min at room temperature. The working dilutions of the glycoprotein-gold complexes are given in Table 1. After two rinses with PBS for 5 min each sections were covered with 1% glutaraldehyde in PBS for 20 min (Roth et al. 1989), rinsed with PBS followed by two rinses with distilled water (5 min each) and air drying. The gold particle signal was amplified using the photochemical silver reaction as described by Taatjes et al. (1987).

For electron microscopical studies, thin sections were incu-

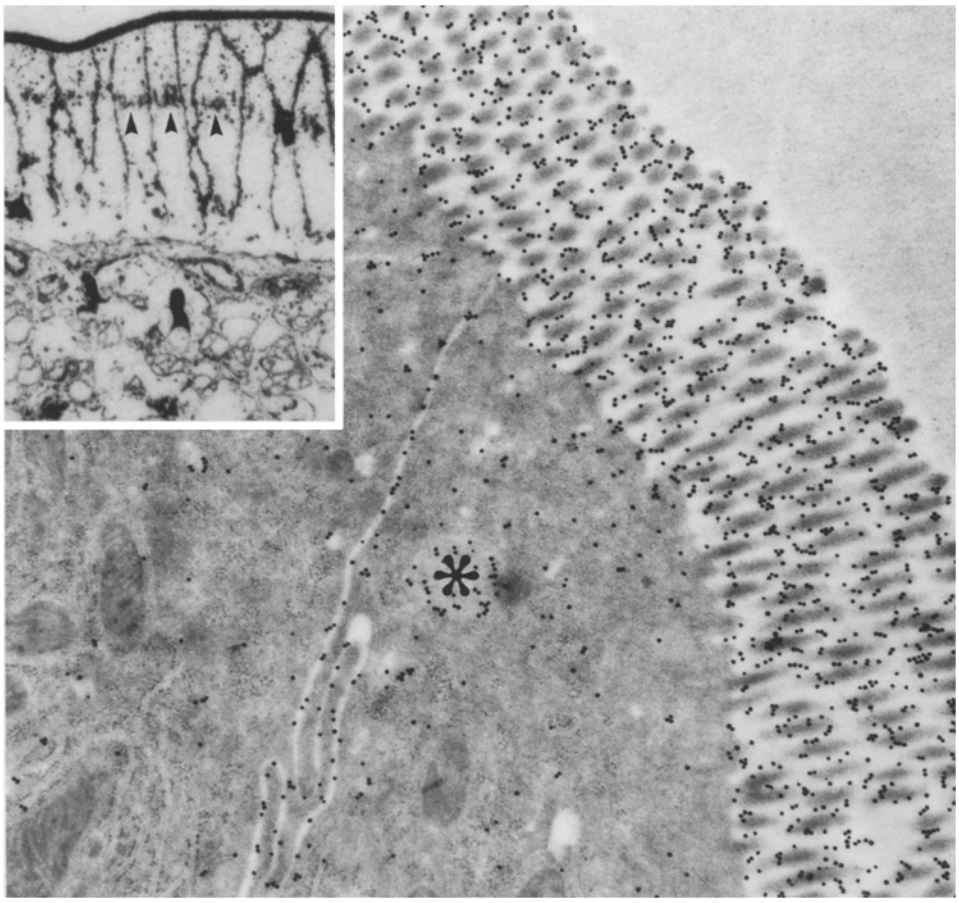


Fig. 7. Rat duodenum, semithin (*inset*) and ultrathin Lowicryl K4M section. At the light microscope level lectin staining is present along the brush border and basolateral plasma membrane, over the Golgi apparatus region (*arrowheads*) and the apical cytoplasm of enterocytes as well as elements of the submucosa (*inset*). By electron microscopy, gold particles can be seen over the brush border, the lateral plasma membrane, a multivesicular body (*asterisk*) and cytoplasmic vesicles. ×23000; ×700 (*inset*)

bated under the same conditions as described for light microscopy with the exception that the glutaraldehyde fixation step and photochemical silver reaction were omitted. Thin sections were contrasted with 3% aqueous uranyl acetate for 4–5 min and lead acetate for 45 s.

Cytochemical controls. The experiments were performed on sections from rat duodenum, colon and kidney and sheep submandibulary gland. Three types of controls were performed: (*a*) sugar and glycoprotein inhibition tests, (*b*) enzymatic pretreatments, and (*c*) β-elimination reaction.

The various monosaccharides and disaccharides were added at concentrations between 5 m*M* and 250 m*M* to the *Datura stramonium* lectin 45 min before use. Unlabeled glycoproteins (10 µg/ml–1.5 mg/ml of ovomucoid, fetuin, asialofetuin, or asialoorosomucoid) were also added to the lectin before use.

Thin sections were pretreated with the following enzymes for 24 h at 37° C: (*i*) neuraminidase (4 U/ml in 0.1 *M* acetate buffer, pH 4.5), (*ii*) β-galactosidase 0.5 U/ml in 0.1 *M* acetate buffer, pH 4.5), (*iii*) endo H (200 mU/ml in 0.5 *M* citrate buffer, pH 5.5), (*iv*) *N*-glycanase (60 U/ml in 0.2 *M* *Tris*-HCl, 2.5 m*M* EDTA, pH 8.6). Positive controls were incubated for the same time period in the appropriate buffer alone. Afterwards, sections were washed in double distilled water (four times 5 min each), and incubated with the *Datura stramonium* lectin followed by ovomucoid-gold (8 nm) complexes as described above.

The β-elimination reaction for the removal of *O*-glycosidically linked oligosaccharide side chains was performed on semithin sections of sheep submandibularly gland. Sections were incubated at 37° C either with 0.2 *N* NaOH for 12 h or with 0.1 *N* NaOH for 24 h. After extensive rinses with distilled water the sections were incubated with the *Datura stramonium* lectin followed by ovomucoid-gold (8 nm) complexes as described above. Pretreated sections were also incubated with the *Helix pomatia* lectin-gold complex as previously described (Roth 1984). This lectin binds to terminal, non-reducing *N*-acetylgalactosamine residues of *O*-glycosidically-linked oligosaccharide side chains and served as a control for the effectiveness of the β-elimination reaction.

The degree of interaction of the ovomucoid-gold complexes with the tissue sections was tested by omitting the lectin incubation step.

Results

Usefulness of the various glycoprotein-gold complexes as second step reagent

This evaluation was performed on thin sections of various tissues and the glycoprotein-gold complexes were rated according to the intensity of staining and the background produced over the same cellular structures. As could be expected from biochemical studies (Broekart et al. 1987), ovomucoid as compared to fetuin, asialofetuin and asialoorosomucoid gave the best staining results and, therefore, was used in all subsequent studies.

Light microscopy

Incubation of semithin sections from the various tissues with the *Datura stramonium* lectin followed by ovomucoid-gold complexes (prepared from 8-nm and 15-nm gold particles) and photochemical silver reaction yielded a sharp, black staining over various cellular and extracellular structures. The results obtained with the use of two different sizes of gold particles were indistinguishable. The pattern and quality of staining obtained is illustrated by a few ex-

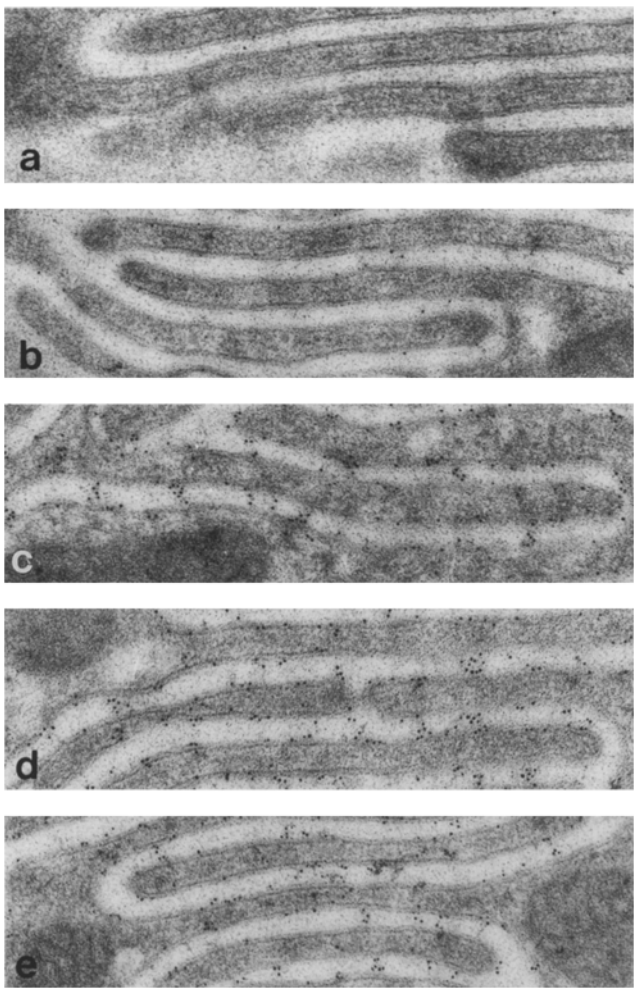


Fig. 8a–e. Basal portion of striated duct epithelia of sheep submandibulary gland, cytochemical control reactions. Addition of *N*-acetyllactosamine the the *Datura stramonium* lectin (**a**) or pretreatment of the sections with endo F (**b**) greatly reduces the lectin staining, whereas addition of *N*-acetylneuraminic acid (**c**), galactose (**d**), and *N*-acetylglucosamine (**e**) does not influence the lectin staining (compare with Figs. 3 and 4). × 55000 (**a**); × 53000 (**b and e**); × 50000 (**c**); × 56000 (**d**)

amples: sheep submandibulary gland (Fig. 1), rat kidney (Fig. 2) and rat duodenum (Fig. 7, inset).

Electron microscopy

For the electron microscopic visualization of lectin molecules bound to reactive configurations on the section surface, ovomucoid-gold complexes prepared from 5-nm, 8-nm and 15-nm gold particle were successfully applied. The staining pattern was not dependent on the use of a particular gold particle preparation. However, when colloidal gold prepared with the tannic acid/citrate technique (Slot and Geuze 1985) was used, the ovomucoid-gold complexes needed to be diluted with PBS containing bovine serum albumin and detergents to overcome nonspecific binding as observed with other glycoprotein-gold and lectin-gold preparations earlier (Taatjes et al. 1987; Roth et al. 1989). Following, only a few examples are given to illustrate the potential of the staining technique. In Figs. 3 and 4, binding of *Datura stramonium* lectin to plasma membrane foldings of striated ducts from sheep submandibulary gland was visualized with ovomucoid-gold complexes prepared from 15-nm and 5-nm gold particles, respectively. Lectin binding was also observed along the plasma membrane, in the Golgi apparatus and the secretion granule limiting membrane of serous acinar cells (Fig. 5). Myoepithelial cells were also positive whereas the mucous acinar cells were not stained. The distribution of lectin binding sites in glomerular capillary loops and proximal tubules of rat kidney is illustrated in Fig. 6. In absorptive intestinal cells, the Golgi apparatus, lysosomal bodies and multivesicular bodies, endocytotic vesicles as well as the basolateral and apical plasma membrane were positive (Fig. 7).

Controls

Among the various mono- and disaccharides used, only *N*-acetyllactosamine (Fig. 8a) and *N,N'*-diacetylchitobiose greatly reduced the staining reaction. Pretreatment of the sections with *N*-glycanase (Fig. 8b) and β-galactosidase also greatly reduced the labeling. Monosaccharides such as *N*-acetylneuraminic acid (Fig. 8c), galactose (Fig. 8d) and *N*-acetylglucosamine (Fig. 8e) at a concentration of 150 m*M* had no appreciable effect on the labeling intensity. The various glycoproteins employed were also inhibitory. As expected, neuraminidase pretreatment resulted in an increase in labeling intensity over various cellular structures. Pretreatment with endo H had no influence on the labeling nor did the β-elimination reaction.

Discussion

In the present study we have shown that the *Datura stramonium* lectin in conjunction with ovomucoid-gold complexes can be used for the specific and high resolution light and electron microscopic detection of lectin binding sites in sections from Lowicryl K4M embedded tissues. Although the family of lectins with a nominal specificity for galactose is quite large, only a few have been previously applied for histochemical purposes (for example see Ellinger and Pavelka 1985; Lucocq and Roth 1984; Roth 1983a, b; Pavelka and Ellinger 1986; Streit et al. 1985; Schulte and Spicer 1983; Kessimian et al. 1986; Vorbrodt et al. 1986; Holthöfer and Virtanen 1987). Furthermore, detailed studies on the structural determinants required for interaction of the galactose-specific lectins with oligosaccharides are available only for the *Ricinus communis* lectin I (Baenziger and Fiete 1979; Debray et al. 1981; Green et al. 1987b) and the *Datura stramonium* lectin (Cummings and Kornfeld 1984; Green et al. 1987a; Yamashita et al. 1987). For the general histochemical detection of β-D-galactosyl residues the *Ricinus communis* lectin I (Nicolson and Blaustein 1972) is most often used.

To our knowledge, no systematic study regarding the usefulness of the *Datura stramonium* lectin as a histochemical reagent has been performed. For both lectins *N*-acetyllactosamine is the primary feature required for oligosaccharide recognition. However, the lectin interaction is strongly modified by other structural features. The *Datura stramonium* lectin has been shown not to interact with dibranched complex-type oligosaccharides regardless of the terminal moiety on the peripheral branches (Green et al. 1987a). In contrast, *Ricinus communis* lectin I was found to interact strongly with dibranched complex-type oligosaccharides (Green et al. 1987b). Furthermore, it is important

to note that the *Ricinus communis* lectin I interaction was not eliminated if α2,6 linked sialic acid residues were present as the terminal moieties. The presence of α2,3 linked sialic acid residues, however, abolished the lectin interaction. Further differences existed in the preference for the type of attachment of the branches to the core mannose residues. The *Datura stramonium* lectin interacts only weakly with C-2,4-branched triantennary sugar chains but strongly with C-2,6-branched triantennary, tetraantennary and bi-, tri- and tetraantennary sugar chains in which at least one *N*-acetyllactosamine repeating unit is present on an outer chain (Yamashita et al. 1987). This principle is applicable only to the complex-type oligosaccharides in which no sialylation and fucosylation of the outer chain moieties has occurred. A bisecting *N*-acetylglucosamine residue in dibranched oligosaccharides and in tribranched or tetrabranched oligosaccharides has no effect on the binding character of the *Ricinus communis* lectin I (Green et al. 1987b) and the *Datura stramonium* lectin (Yamashita et al. 1987), respectively. These biochemical investigations have clearly shown that both lectins are able to distinguish among a large number of closely related oligosaccharides. With these findings in mind, both lectins can be employed as effective tools for the histochemical analysis of complex-type, asparagine linked oligosaccharide chains.

Acknowledgments. We thank Marlis Kasper for skillful technical assistance and Daniel Wey for preparing the photographs and Prof. G. Molineux and his colleagues (Brisbane, Australia) for providing the sheep tissue.

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