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Mucins in cat airway secretions

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Mucous secretions were obtained from cat tracheas that had received [^{3}H]glucose and [^{35}S]sulphate to radiolabel mucus glycoproteins biosynthetically. Samples were collected under resting ('basal') conditions as well as after pilocarpine stimulation and were separated into gel and sol phases by centrifugation. Macromolecules were partially purified by using gel chromatography on Sepharose CL-4B, and the species that were eluted with the void volume were then separated into two major populations with isopycnic density-gradient centrifugation in CsCl. The major component from the gel phase of pilocarpine-induced secretions had a buoyant density typical of mucins and was observed as linear and apparently flexible chains by electron microscopy. Reduction of disulphide bonds gave subunits that could be further cleaved by trypsin digestion into components of approximately the same size as the high-M_r glycopeptides obtained from other mucins after this treatment. In contrast, the dominant species in the gel phase of the 'basal' secretion had a significantly higher buoyant density than expected for mucins and was largely unaffected by reduction, as studied by gel chromatography. The macromolecules were fragmented by trypsin, suggesting that they contain a polypeptide backbone. This more dense component also predominated in the sol phase both from the 'basal' secretions and from the pilocarpine-released secretions. Digestion with DNAase, chondroitin ABC lyase or heparan sulphate lyase had no effect, which shows that this component is not DNA, a dermatan sulphate/chondroitin sulphate or a heparan sulphate proteoglycan. In contrast, endo-β-galactosidase and keratanase caused some fragmentation, suggesting that the molecules contain some linkages of the poly-(*N*-acetyl-lactosamine) type, although the degradation was not as extensive as expected for keratan sulphate. Treatment with alkaline borohydride resulted in extensive fragmentation of the high-M_r glycopeptides from both components, indicating that the glycans were oligosaccharides that were probably *O*-linked. The monosaccharide compositions of both components were consistent with that expected for mucins. The data are in keeping with the major component from the pilocarpine-stimulated gel secretions being a mucus glycoprotein and the more dense component being a mucin-like molecule, possibly related to the keratanase-sensitive material isolated from canine trachea by Varsano, Basbaum, Forsberg, Borson, Caughey & Nadel [(1987) Exp. Lung Res. **13**, 157–184].

INTRODUCTION

In the airways, mucus glycoproteins (mucins) are produced by a number of different secretory cell types, including the submucosal glands and the globlet cells interspersed among the ciliated cells in the surface epithelium (Jeffery, 1978; Jones, 1978; Nadel *et al.*, 1985). The chemical nature of these macromolecules and the relative contribution to mucus by the different cell types may be important determinants of the physical properties of the gel and thus the ability of secreted mucus to perform its functions. Heterogeneity of mucins from the different cell types has been identified by both histochemical and biochemical means (Jeffery, 1978; Gallagher *et al.*, 1986; for a review see, e.g. Reid *et al.*, 1982).

Two precursors of mucins, [^{3}H]glucose and [^{35}S]sulphate, incorporated biosynthetically into cat tracheal secretions, are released in different proportions by different stimuli (Gallagher & Richardson, 1982; Gallagher *et al.*, 1986). Unstimulated ('basal') secretions contain material labelled with both radioisotopes, whereas cholinergic agonists, such as pilocarpine, cause secretion of mucins rich in ^{35}S that apparently originate from the submucosal glands (Florey *et al.*, 1932; Jeffery, 1978). Irritants such as NH_3 vapour and bacterial toxins such as rhamnolipid from *Pseudomonas aeruginosa* release ^{3}H-rich macromolecules, possibly from the surface epithelium (Gallagher *et al.*, 1986; Somerville *et al.*, 1988; Davies *et al.*, 1990). Previous investigations carried out on the high-M_r components in 'basal' and pilocarpine-induced secretions indicated that they were largely composed of mucus glycoproteins, but the analyses were not sufficiently detailed for a satisfactory comparison to be made with mucus glycoproteins isolated from the mucosal epithelia of larger mammals, including man. In the present paper we compare macromolecules from the 'basal' secretion with those obtained after stimulation with pilocarpine, a cholinergic agonist, to elicit secretion from the submucosal glands. Two major and distinctive high-M_r glycoconjugate components were identified, but only one of these behaves as a 'typical' mucin.

EXPERIMENTAL

Materials

Specific-pathogen-free cats were obtained from an accredited breeder. Pentobarbitone sodium (Sagatal) was purchased from May and Baker, and pilocarpine, DNAase I (EC 3.1.21.1; type IV), trypsin (EC 3.4.21.4; type XIII, *N*-tosyl-L-phenylalanyl-chloromethane-treated) and papain (EC 3.4.22.2; type III) were from Sigma Chemical Co. Sepharose CL-4B, Sepharose CL-2B and Superose 6 HR10/30 were from Pharmacia, and conc. NH_3 (sp. gr. 0.88) was from Fisons. D-[1-^{3}H]Glucose and $Na_2{}^{35}SO_4$, both carrier-free, were brought from Amersham International. Chondroitin ABC lyase (EC 4.22.4), heparan sulphate lyase (EC 4.2.2.8) and keratanase from *Pseudomonas* sp. IFO-13309 were products of Seikagaku Kogyo Co. (Tokyo, Japan). Endo-

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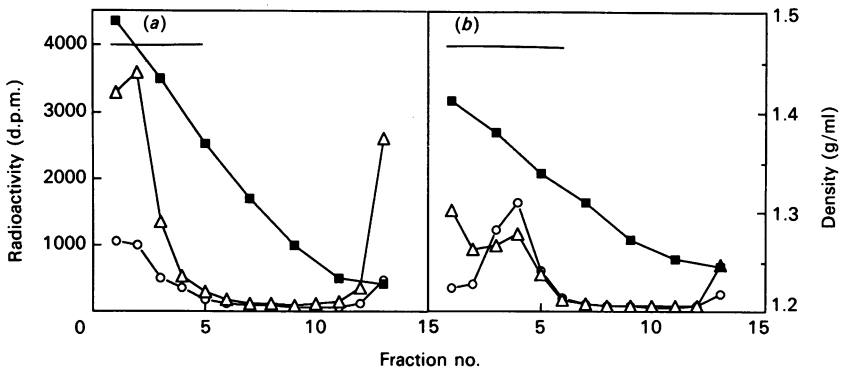

Fig. 1. Isopycnic density-gradient centrifugation in CsCl/4M-guanidinium chloride of cat tracheal mucins from the gel phase of (*a*) 'basal' secretion (*b*) pilocarpine-released material

Mucus samples were separated into gel and sol phases, dialysed into 4 M-guanidinium chloride and chromatographed on Sepharose CL-4B. Material that was eluted with the void volume was pooled and subjected to density-gradient centrifugation in CsCl/4 M-guanidinium chloride (36000 rev./min for 65 h at 15 °C in the 70.1 Ti rotor, of the Beckman L5–65B ultracentrifuge). △, ^3H radioactivity; ○, ^{35}S radioactivity; ■, density. Recoveries: (*a*) ^3H, 78%, and ^{35}S, 82%; (*b*) ^3H, 76%, and ^{35}S, 85%. Fractions were pooled as shown by the horizontal bars and subjected to density-gradient centrifugation in CsCl/0.2 M-guanidinium chloride.

β-galactosidase from *Escherichia freundii* was from ICN Biomedicals.

Collection of radiolabelled tracheal secretions

Anaesthesia was induced by intraperitoneal injection of pentabarbitone sodium (42 mg/kg) and maintained by subsequent doses through a femoral-vein catheter. One cut was made in the trachea just caudal to the larynx, another as far caudally in the neck as possible. Two cannulae were tied into the latter, one through which the animal breathed, the other, together with a cannula in the rostral cut, being used to isolate a tracheal segment from which mucins could be collected at intervals. The latter remained filled with Krebs–Henseleit solution (Krebs & Henseleit, 1932) during most of the experiment. For further details see Gallagher *et al.* (1975).

In order to deplete the mucin stores and allow the cells to replenish with newly synthesized radiolabelled macromolecules, tracheal segments were filled with Krebs–Henseleit solution containing pilocarpine (25 μM) for 45 min to cause secretion from the submucosal glands (Florey *et al.*, 1932; Jeffery, 1978). This solution was expelled with 20 ml of air from a syringe, and 300 ml of NH_3 vapour (300 ppm) was passed through the tracheal segment over 10 min to stimulate release of macromolecules from the surface epithelium (Florey *et al.*, 1932). The segment was again filled with Krebs–Henseleit solution for 5 min before being flushed through to remove this material.

$Na_2{}^{35}SO_4$ (2 mCi) and [^3H]glucose (0.5 mCi) in 4 ml of Krebs–Henseleit solution were introduced into the tracheal segment and left there for 3 h during re-synthesis of mucins before being washed out with 2 × 6 ml of Krebs–Henseleit solution and discarded. Mucins from the 'basal' secretion were then collected in the unstimulated trachea for 2 h and flushed out with 6 ml of Krebs–Henseleit solution containing pilocarpine (25 μM). After 1 h of pilocarpine stimulation the trachea was flushed with the same volume of Krebs–Henseleit solution and the washings were retained. This sample is referred to below as the pilocarpine-induced secretion. Samples from six animals were pooled.

Isolation of mucus glycoproteins

The mucous secretions, collected in Krebs–Henseleit solution, were centrifuged at 36000 rev./min for 20 min at 3 °C in a Beckman LS-50 centrifuge to separate the gel and sol phases. The sol phase was mixed with 6 M-guanidinium chloride/5 mM-Na_2EDTA/5 mM-*N*-ethylmaleimide/0.1 mM-phenylmethanesulphonyl fluoride/10 mM-sodium phosphate buffer, pH 6.5, to give a final concentration of 4 M-guanidinium chloride. The gel samples were solubilized in 4 M-guanidinium chloride/5 mM-Na_2EDTA/5 mM-*N*-ethylmaleimide/0.1 mM-phenylmethanesulphonyl fluoride/10 mM-sodium phosphate buffer, pH 6.5. The samples were concentrated, if necessary, in an Amicon ultrafiltration cell (PM 10; cut-off M_r 10000), dialysed against 4M-guanidinium chloride / 5 mM-Na_2EDTA / 10 mM-sodium phosphate buffer, pH 6.5, and chromatographed on a Sepharose CL-4B column (80 cm × 3.2 cm) eluted at 32 ml/h with the same solution. Portions (200 μl) of the fractions (7 ml) were mixed with 5 ml of scintillation fluid (Ready Safe; Beckman Instruments) and counted in a β-radiation scintillation counter (LKB 1214 Rackbeta). Correction to d.p.m. was made by using an automatic procedure based upon an external quench standard.

High-M_r glycoproteins, which chromatographed with the void volume on Sepharose CL-4B, were concentrated by ultrafiltration and dialysed against 6 M-guanidinium chloride / 5 mM-Na_2EDTA/10 mM-sodium phosphate buffer, pH 6.5. CsCl and 5 mM-Na_2EDTA/10 mM-sodium phosphate buffer, pH 6.5, were added to give an initial density of approx. 1.40 g/ml and subjected to isopycnic density-gradient centrifugation in a Beckman L5-65B ultracentrifuge (70.1 Ti rotor; 36000 rev./min for approx. 65 h at 15 °C). Tubes were emptied from the bottom and samples (100–300 μl) were taken from determination of radioactivity. The density was measured with a Carlsberg pipette as a pycnometer. The denser fractions were pooled (see Fig. 1) and dialysed against 0.2 M-guanidinium chloride / 5 mM-Na_2EDTA/10 mM-sodium phosphate buffer, pH 6.5. CsCl and buffer were added to a density of 1.50 g/ml, and the macromolecules were subjected to a second density-gradient run under the conditions outlined above.

Degradative methods

Subunits were obtained after reduction of disulphide bonds in 6 M-guanidinium chloride / 5 mM-Na_2EDTA / 10 mM-dithiothreitol / 10 mM-Tris/HCl buffer, pH 8.0, for 5 h at 37 °C. Subsequent alkylation was achieved by adding a 2.5 molar excess of iodoacetamide over dithiothreitol and leaving the sample for at least 12 h in the dark. Tryptic digestion (100 μg of enzyme/ml) was performed at 37 °C for 5 h after dialysis against 0.1 M-Tris/HCl buffer, pH 8.0. Digestion with DNAase (100 μg of enzyme/ml) was performed at 37 °C for 5 h after dialysis against

50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-$MgCl_2$. Samples were then analysed on a Sepharose CL-2B column (80 cm × 1.6 cm). Glycosidase digestions were performed on high-M_r glycopeptides obtained with papain digestion of the two components (100 μg of enzyme/ml in 50 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-Na_2EDTA and 5 mM-cysteine hydrochloride for 12 h at 65 °C). The papain was inactivated by alkylation (2.5 molar excess of iodoacetamide over papain, for 12 h at 37 °C in the dark), and the samples were dialysed against 0.1 M-ammonium acetate buffer, pH 7.0, before being freeze-dried. Portions were then redissolved in the appropriate buffers and digested with chondroitin ABC lyase (5 units, for 12 h at 37 °C in 0.1 M-Tris/acetate buffer, pH 7.3, containing 10 mM-Na_2EDTA), heparan sulphate lyase (0.1 munit, for 12 h at 37 °C in 50 mM-Tris/acetate buffer, pH 7.0, containing 5 mM-calcium acetate), endo-β-galactoside (1 munit and 10 munits for 12 h at 37 °C in 50 mM-sodium acetate buffer, pH 5.6) and keratanase (0.01 munit for 12 h at 37 °C in 50 mM-Tris/HCl buffer, pH 7.4). Samples were then chromatographed on a Superose 6 HR10/30 column eluted with 0.1 M-ammonium acetate buffer, pH 7.0, at 0.25 ml/min. Fractions (0.5 ml) were taken for determination of radioactivity by β-radiation scintillation counting. Alkaline borohydride treatment of both components was performed after the addition of equal volumes of water and 2 M-$NaBH_4$/0.1 M-NaOH (Carlson, 1968). After 16 h at 45 °C, acetic acid was added and the samples were chromatographed on a Superose 6 column under the conditions outlined above.

Monosaccharide analysis

Samples were hydrolysed with 4 M-trifluoroacetic acid under N_2 for 2 h at 100 °C. The acid was evaporated in a stream of N_2, the samples were redissolved in distilled water and portions were subjected to ion-exchange chromatography on either a CarboPac PA1 column (Dionex) eluted with 8 mM-NaOH at 1 ml/min at 20 °C or an Aminex HPX-87P column (Bio-Rad Laboratories) eluted with water at 0.25 ml/min at 45 °C. Fractions (1 ml and 0.5 ml respectively) were collected and their radioactivities counted in a β-radiation scintillation counter. The hydrolysis conditions for fucose were studied by using high-M_r glycopeptides from rat small intestine. A similar yield was obtained after 1 h and after 2 h in 4 M-trifluoroacetic acid as well as after 2 h in 2 M-trifluoroacetic acid, suggesting that no extensive degradation occurs under the conditions used here.

Electron microscopy

Specimen preparation for electron microscopy was performed essentially as described by Sheehan *et al.* (1986). Stock solutions of the mucins (concentration unknown) in 6 M-guanidinium chloride were diluted 10–100-fold with this solvent or with 50 mM-magnesium acetate, depending on the hypophase used. In brief, samples were spread in monolayers of benzyldimethylalkylammonium chloride, collected on to carbon-coated grids and examined in the electron microscope after staining with uranyl acetate and/or shadowing with platinium/carbon. Electron microscopy was performed with a JEOL 100CX instrument at 80 or 100 keV. Length distributions were measured on prints (final magnification × 30000–40000) with a Planix 5000 digitizing area/line planimeter (Hall and Watts, Lynx Trading Estate, Yeovil, Somerset, U.K.).

RESULTS AND DISCUSSION

In the 'basal' secretions most of the radiolabelled material (^3H, 62%; ^{35}S, 66%) was in the sol phase, whereas the reverse was true for the pilocarpine-released material (^3H, 41%; ^{35}S, 42%). The gel and sol phases from both secretions contained major peaks that were eluted with the void volume of Sepharose CL-4B, as expected for undegraded mucins. In addition, various amounts of smaller, partially included, components were present (results not shown). In all cases, recovery of both ^3H and ^{35}S was greater than 90%.

When the void-volume material from the Sepharose CL-4B column (from the four samples) was subjected to isopycnic density-gradient centrifugation in CsCl/4 M-guanidinium chloride, the major components were recovered between the densities of 1.33 g/ml and 1.48 g/ml. Similar results were obtained for both the gel and the sol phases of the 'basal' and pilocarpine-induced secretions; those from the gel phases are shown in Figs. 1(*a*) and 1(*b*). The 'basal' secretion, in particular, contained ^3H-labelled material at a density < 1.30 g/ml, as expected for serum-type and non-glycosylated proteins as well as lipids. This observation shows that non-mucin components could be large enough to appear in the void volume of Sepharose CL-4B and thus that pure mucins are not likely to be obtained after a single chromatography step on such (or similar) gels. When material with a buoyant density > 1.33 g/ml was centrifuged in CsCl/0.2 M-guanidinium chloride the samples collected under 'basal' conditions and those obtained after pilocarpine stimulation showed distinct and characteristic patterns of separation (Fig. 2). In the gel and the sol phases from the 'basal' secretion (Figs. 2*a* and 2*b*) and the sol phase from the pilocarpine-released material (Fig. 2*d*) a major peak with a high $^3H/^{35}S$ ratio was found at a density of 1.62 g/ml, higher than expected for mucus glycoproteins. Less prominent peaks, with a lower $^3H/^{35}S$ ratio, occurred at density of 1.50 g/ml. In contrast, the gel phase from the pilocarpine-induced secretion was dominated by a peak with a lower $^3H/^{35}S$ ratio at a density of 1.50 g/ml, as expected for mucus glycoproteins (Fig. 2*c*), and only a minor component, with a high $^3H/^{35}S$ ratio, was found at a density of 1.62 g/ml. Recoveries in all cases were greater than 50%.

Gel chromatography on Sepharose CL-2B showed that both major components obtained after density-gradient centrifugation appeared mainly in the void volume of the column (Figs. 3*a* and 3*d*). After reduction, however, the two components behaved differently. The macromolecules with a buoyant density of 1.50 g/ml, released in response to pilocarpine stimulation, were completely fragmented into subunits, as expected for mucins (see, e.g., Carlstedt *et al.*, 1983*b*), whereas those with a buoyant density of 1.62 g/ml from the 'basal' secretion were largely unaffected, at least to the extent that the elution profile did not change (Figs. 3*b* and 3*e*). Both components were sensitive to tryptic digestion, and high-M_r glycopeptides were obtained (Figs. 3*c* and 3*f*). Digestion with DNAase caused no fragmentation of the 'high-density' component (results not shown). The 'fragmentation pattern' for the less dense macromolecules from the pilocarpine-released material was very similar to that observed for cervical mucins (Carlstedt *et al.*, 1983*b*), gastric mucins (Carlstedt & Sheehan, 1984), mucins from 'normal' human respiratory secretions (Thornton *et al.*, 1990) and mucins obtained from chronic-bronchitic and cystic-fibrotic sputum (I. Carlstedt, J. K. Sheehan & D. J. Thornton, unpublished work).

To investigate the carbohydrate portions of the two components further high-M_r glycopeptides from each (Figs. 4*a* and 4*e*) were digested with chondroitin ABC lyase and heparan sulphate lyase, endo-β-galactosidase and keratanase before chromatography on Superose 6. Chondroitin sulphate lyase and heparan sulphate lyase caused no degradation (results not shown), and it is concluded that neither component is a proteoglycan with chondroitin sulphate/dermatan sulphate or heparan sulphate side chains. The 'high-density' component appears therefore to be distinct from airway glycoconjugates of

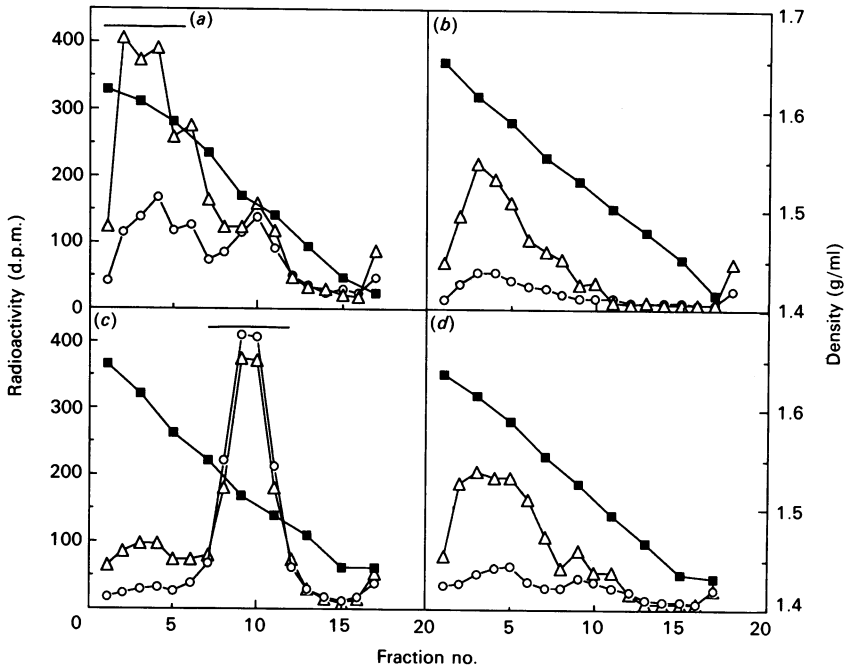

Fig. 2. Isopycnic density-gradient centrifugation in CsCl/0.2M-guanidinium chloride of cat tracheal mucins from the gel phase (*a* and *c*) and sol phase (*b* and *d*) of the 'basal' (*a* and *b*) and pilocarpine-induced (*c* and *d*) secretions

Fractions from the CsCl/4 M-guanidinium chloride gradient were pooled as shown in Fig. 1 and re-centrifuged in CsCl/0.2 M-guanidinium chloride as described in the legend to Fig. 1 △, ^{3}H radioactivity; ○, ^{35}S radioactivity; ■, density. Recoveries: (*a*) ^{3}H, 99%, and ^{35}S, 88%; (*b*) ^{3}H, 52%, and ^{35}S, 52%; (*c*) ^{3}H, 95%, and ^{35}S, 75%; (*d*) ^{3}H, 103%, and ^{35}S, 67%. Material was pooled as indicated by the horizontal bars.

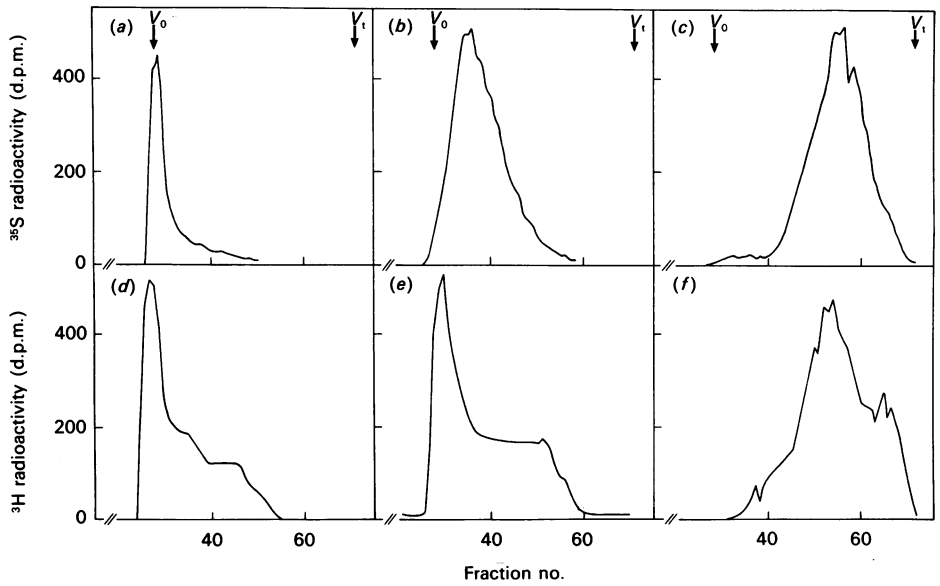

Fig. 3. Gel chromatography on Sepharose CL-2B of the 'low-density' component (*a–c*) and 'high-density' component (*d–f*) and fragments thereof

The macromolecules were isolated after gel chromatography and isopycnic density-gradient centrifugation as described in the text. (*a*) and (*d*) Non-degraded macromolecules; (*b*) and (*e*) macromolecules after reduction; (*c*) and (*f*) macromolecules after tryptic digestion (*c*) of reductive fragments of pilocarpine-released material and (*f*) of non-degraded molecules from the 'basal' secretion. The 'low-density' component was monitored as ^{35}S radioactivity, the 'high-density' component as ^{3}H radioactivity. Recoveries were in all cases greater than 90%.

similar buoyant density reported by Bhaskar *et al.* (1986), which were largely chondroitin sulphate. Both components were, however, partially degraded by endo-β-galactosidase (Figs. 4*b* and 4*f*) and keratanase (Figs. 4*c* and 4*g*) indicating that they contain some poly-(*N*-acetyl-lactosamine)-type linkages. A second treatment with 10 times the initial concentration of endo-β-galactosidase did not change the pattern of degradation, indicating that fragmentation was complete under the original conditions. The elution pattern observed after digestion with endo-β-galactosidase was broadly similar to that seen with keratanase, although the low-M_r fragments obtained with keratanase appeared to be somewhat smaller than with endo-β-galactosidase. The digestions were performed on high-M_r glycopeptides in order to decrease the possibility that any fragmentation observed was due to spurious proteinase activity in the enzyme preparations used. Removal of the side chains of

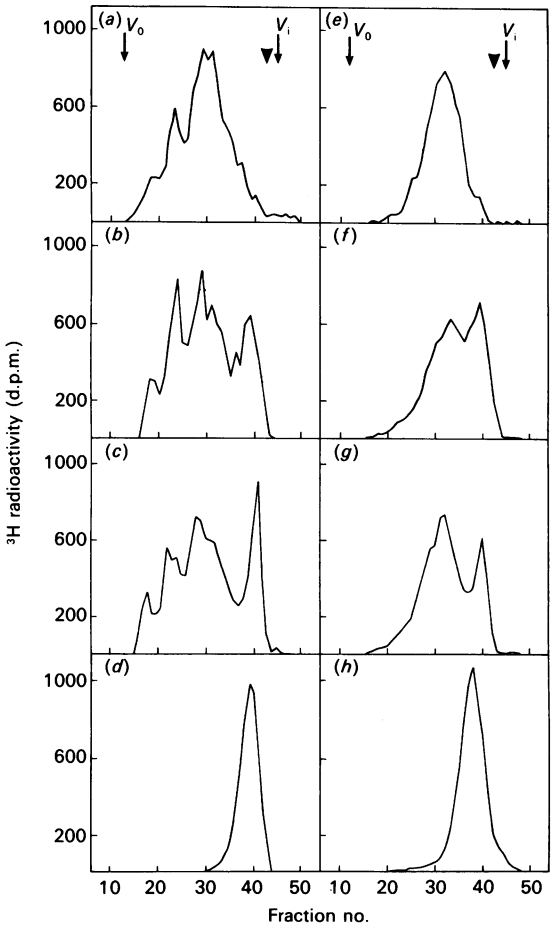

Fig. 4. Gel chromatography on Superose 6 of high-M_r glycopeptides from the 'low-density' component (*a–d*) and 'high-density' component (*e–h*) before (*a* and *e*) and after treatment with endo-β-galactosidase (*b* and *f*), keratanase (*c* and *g*) and alkaline borohydride (*d* and *h*)

High-M_r glycopeptides of the 'low-density' component and 'high-density' component were prepared by papain digestion as described in the text. All material was monitored as ^{3}H radioactivity. Recoveries were in all cases greater than 80%. The totally included volume (V_i) was determined with ^3H_2O. The elution position of lactose is indicated by the arrowhead.

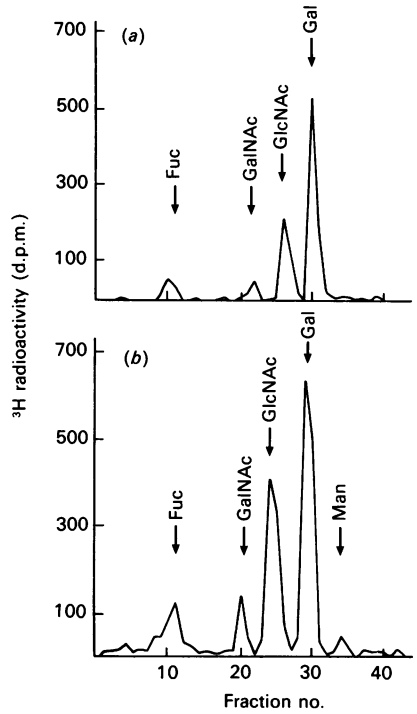

Fig. 5. Monosaccharide analysis of the 'low-density' component (*a*) and 'high-density' component (*b*)

Samples were hydrolysed and subjected to ion-exchange chromatography on a CarboPac PA1 column. Arrows indicate the expected elution positions of fucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose and mannose. Recoveries were greater than 50%.

both components after treatment with alkaline borohydride (Figs. 4*d* and 4*h*) indicated that the glycans were oligosaccharides rather than polysaccharides. However, the chromatographic behaviour of lactose and ^3H_2O shows that the resolution of the gel is not sufficient to determine the size of the oligosaccharides and to allow comparison with the smallest fragments obtained with keratanase and endo-β-galactosidase digestion.

Analysis of the monosaccharide components of the side chains showed that both components contained fucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine, sugars typical for mucus glycoproteins (Fig. 5*a*). Possibly, the 'high-density' component also contained a little mannose (Fig. 5*b*). Since glucose and galactose do not separate well on the CarboPac PA1 column, the presence of galactose rather than glucose in the macromolecules was verified by chromatography on an Aminex HPX-87P column (results not shown).

The size and macromolecular structure of the major 'low-density' component from the gel phase of the pilocarpine-induced secretion was further studied by electron microscopy. In the first set of experiments, the samples were rapidly diluted in one step into 50 mM-magnesium acetate before spreading. With this procedure the molecules were observed as tangled structures, likely to contain more than one molecule (Fig. 6*a*). After gradual stepwise dilution or dialysis into this solvent, the mucins appeared as a mixture of such 'aggregates' and long filamentous threads (results not shown). Spreading on 6 M-guanidinium chloride further decreased the proportion of 'aggregates' and almost all molecules were observed as linear structures (Fig. 6*b*). The distribution of contour lengths was very broad (Fig. 7), with number-average and weight-average lengths of 1.6 μm and 3.7 μm respectively. The distribution ranged from about 0.2 μm to approx. 9 μm, and the data are, apart from a significant 'subpopulation' at 5–7 μm, much in keeping with previous studies on respiratory mucins (Jenssen *et al.*, 1980; Rose *et al.*, 1984; Slayter *et al.*, 1984; Mikkelsen *et al.*, 1985; Sheehan *et al.*, 1986; Marianne *et al.*, 1987) as well as on pig gastric and human cervical mucins (Sheehan *et al.*, 1986).

GENERAL DISCUSSION

The radioactive precursors, [^{35}S]sulphate and [^{3}H]glucose, used in the present study bring about different labelling patterns in the tracheal epithelium. [^{35}S]Sulphate is principally found in the submucosal gland cells, whereas much radioactivity from [^{3}H]glucose becomes located in the apical border of the surface epithelium (Jeffery, 1978; Davies *et al.*, 1990). Since pilocarpine elicits a specific secretory response in the submucosal glands, it is reasonable to conclude that the major part of the ^{35}S-labelled macromolecular components present in such secretions originate from these glands. Although a major proportion of [^{3}H]glucose is taken up by the surface epithelium, the origin of the 'high-density' component is less certain.

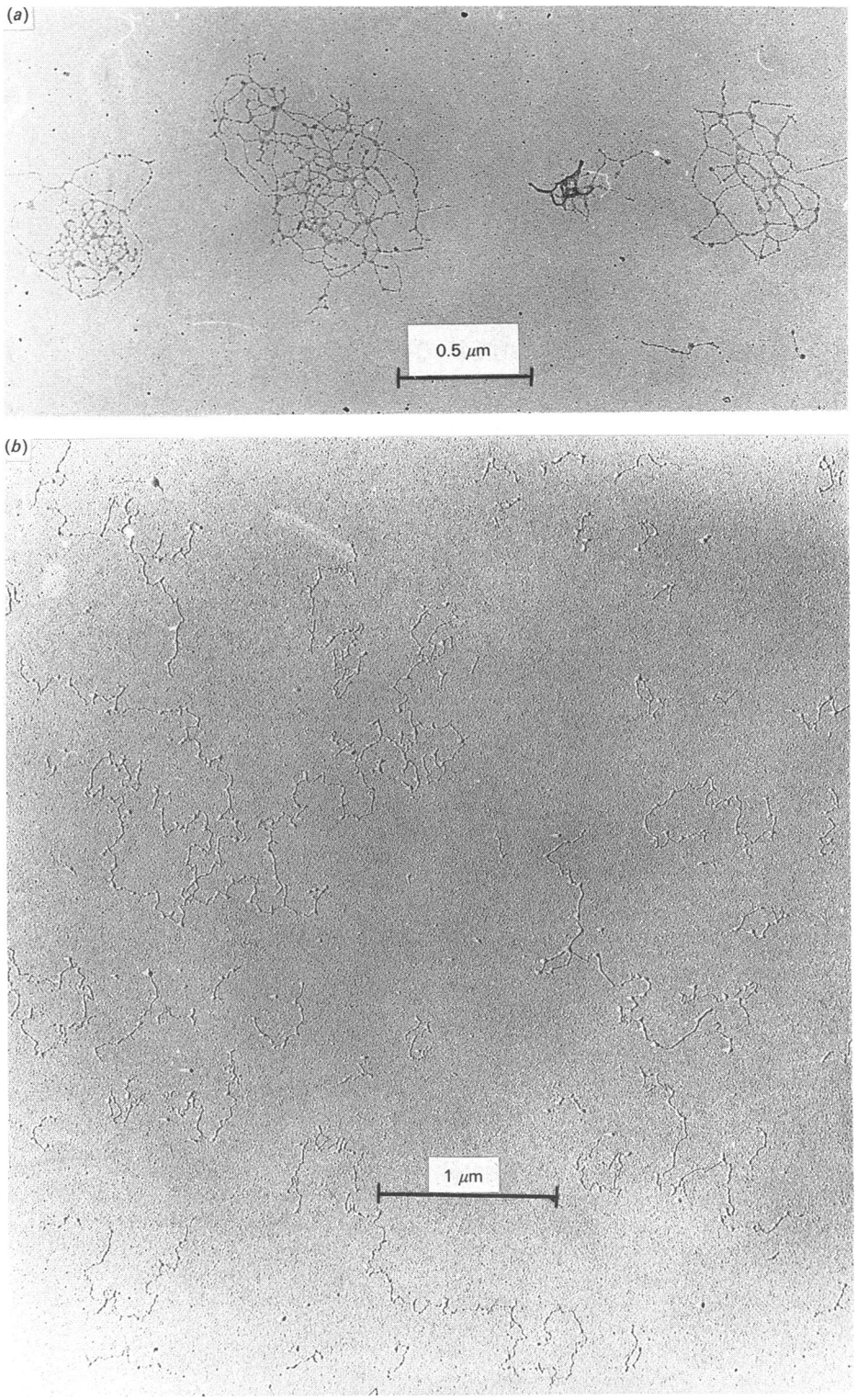

Fig. 6. Electron microscopy of the 'low-density' component

The macromolecules were spread in a monolayer of benzyldimethylalkylammonium chloride, picked up on to carbon-coated grids and treated with uranyl acetate and/or shadowed with platinum/carbon. (*a*) Mucins rapidly diluted from 6 M-guanidinium chloride into 50 mM-$MgCl_2$ appeared as 'aggregates'. (*b*) When spread directly on to 6M-guanidinium chloride, the molecules were observed mainly as relaxed filamentous structures.

The results of the gel chromatography on Sepharose CL-4B of 'basal' and pilocarpine-induced secretions agree with those obtained by Gallagher *et al.* (1986). All samples contained high-M_r material excluded on the gel and partially included species. Isopycnic density-gradient centrifugation was used to separate the material excluded on Sepharose CL-4B into two different major populations. Although both components were present in the 'basal' and the pilocarpine-released material, the proportions varied. The gel and sol phases from the 'basal' secretions and the sol phase from the pilocarpine-induced secretion contained a

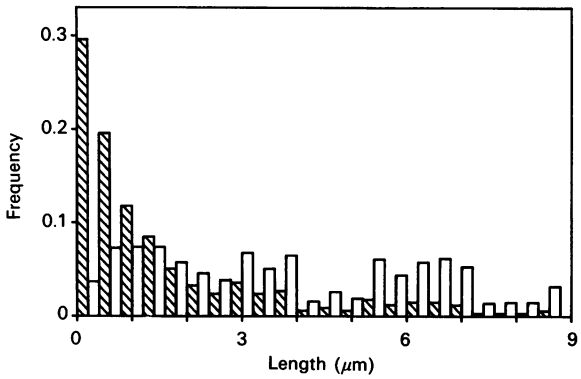

Fig. 7. Distribution of contour lengths for the 'low-density' component

The contours of 330 molecules were traced with a digitizing planimeter as described in the text. The number-average contour length (l_n) was 1.6 μm and the weight-average contour length (l_w), calculated assuming that the length is directly proportional to M_r, was 3.7 μm. ▨, Number distribution; □, weight distribution.

major 'high-density' component with a high $^3H/^{35}S$ ratio and a less prominent 'low-density' one with a lower $^3H/^{35}S$ ratio. However, the gel phase of the pilocarpine-released material the proportions were reversed. These results show that, in terms of radiolabelled macromolecular components (excluded from Sepharose CL-4B), the differences in the gel and sol phases lay in the proportions rather than the nature of the constituents.

The buoyant density of the predominant ^{35}S-labelled component in the pilocarpine-induced secretions was 1.50 g/ml, which is similar to that of mucins from other sources (Carlstedt *et al.*, 1983*a*; Thornton *et al.*, 1990). Also, the size of subunits obtained after reduction of disulphide bonds is in keeping with data for other mucins isolated after slow stirring in a denaturing solvent (Carlstedt & Sheehan, 1984; Thornton *et al.*, 1990). The size of the major fragments (T-domains) obtained after tryptic digestion of subunits suggests that large 'protected' stretches of the protein core are flanked by segments susceptible to proteolysis, which is also a characteristic feature of mucins. The side chains are oligosaccharides, which are probably *O*-linked, and contain sugars typical for mucus glycoproteins. The partial fragmentation observed with endo-β-galactosidase and keratanase shows that the glycans contain some poly-(*N*-acetyllactosamine)-type linkages. Finally, the undegraded macromolecules were observed as linear and apparently flexible structures with electron microscopy. These observations, together with the fact that the macromolecules are released after stimulation with pilocarpine, lead us to conclude that they represent a mucus glycoprotein originating from the submucosal glands.

In contrast, the major ^3H-labelled component in the 'basal' secretion does not behave as a 'typical' mucin. The buoyant density is too high and the molecules are not susceptible to reduction to the extent usually observed. The component appears to be a glycoprotein because it is sensitive to tryptic digestion. No effect was noted by chondroitin ABC lyase or heparan sulphate lyase, which suggests that the molecule is not substituted with dermatan sulphate/chondroitin sulphate or heparan sulphate. The molecules are mucin-like in that they contain oligosaccharides rather than polysaccharides and have a monosaccharide profile consistent with that expected for mucins. The strong pulse-labelling with [^{3}H]glucose suggests a rapid metabolic turnover, whereas the mucin stores in the mucosal glandular cells are likely to have a lower turnover rate with most of the material retained in the secretory granules unless an appropriate stimulus is experienced. It is possible that the component is related to the keratanase-sensitive macromolecule secreted by epithelial cells cultured from the dog airways (Varsano *et al.*, 1987), although the fragmentation seen with keratanase was not to the extent expected for keratan sulphate. The origin of the component is not known, but indirect evidence suggests the apical border of the mucosal surface.

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