Physicochemical properties of avian tracheal mucus

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Dual-radiolabelled avian tracheal secretions were obtained by giving $\text{Na}_2^{35}\text{SO}_4$ and $\text{D-[1-}^3\text{H]glucosamine}$ simultaneously into the lumen of the trachea in preparations *in vitro*. These secretions comprised fibrillar, gelatinous and soluble-phase mucins. These were eluted as single components in the non-retarded fractions from Bio-Gel A-15m. Although no evidence of the presence of subunit structure was found, chemical and radiolabelling analyses showed a high degree of internal inhomogeneity among the three types of mucins. The differences among these mucins could be attributed to the chemical nature of their constituent glycoproteins. Glycoprotein fractions separated by ion-exchange chromatography were found to contain sulphate and *N*-acetylneuraminic acid residues in differing amounts. The overall acidic properties appeared to be correlated with ester sulphate content. A close similarity in the carbohydrate composition and a reciprocal relationship between the total ester sulphate residue contents and ^{35}S - and ^3H -labelling suggested that, in addition to stepwise glycosylation and sulphation, some pre-existing sulphated oligosaccharides might have been utilized for the synthesis of acidic glycoproteins.

Various physicochemical properties of mucus material from gastrointestinal, cervical, tracheo-bronchial and nasal tracts of a variety of mammalian systems and of human sputum had been investigated previously (for review articles see Gottschalk, 1972; Elstein & Parke, 1977). However, little work had been performed on tracheal mucus secretions, which were collected either by perfusion techniques *in vivo* (Gallagher *et al.*, 1975; Phipps *et al.*, 1977) or from tracheal explants in organ culture (Kent *et al.*, 1969; Boat *et al.*, 1971; Ellis & Stahl, 1973). Both these techniques had disadvantages. Either secretions were collected under nervous and hormonal influences, or the luminal secretions were mixed with the products released from the subluminal surfaces. To our knowledge, no detailed investigation on the physicochemical aspects of avian tracheal mucus has been conducted so far. The present study was undertaken to characterize some physicochemical aspects of mucus secretion obtained from the luminal surface of the avian trachea (Balfre & Richardson, 1978), and also to investigate the relationship between the physical properties and chemical structure of these mucins.

Materials and methods

Materials

Male and female chickens (12–30 weeks old) were

obtained from Orchard Farm, Little Kingshill, Bucks., U.K. DEAE-cellulose (DE-52), Ultrogel AcA-22 and Bio-Gel A-type gel-filtration resins were obtained from Whatman, Maidstone, Kent, U.K., from LKB Instruments, South Croydon, Surrey, U.K., and from Bio-Rad Laboratories, Watford, Herts., U.K., respectively. Gentamicin, penicillin, streptomycin and fungizone were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. Papain (type III, $2 \times$ crystallized from *Papaya* latex), Pronase (proteinase type VI, from *Streptomyces griseus*) and their insolubilized forms attached to CM-cellulose, neuraminidase (type V, from *Clostridium perfringens*) and its insolubilized form attached to beaded agarose, and arylsulphatase (type H-1, from *Helix pomatia*) were all obtained from Sigma Chemical Co., Poole, Dorset, U.K. $\text{Na}_2^{35}\text{SO}_4$ (25–40 Ci/mg), $\text{D-[1-}^3\text{H]glucosamine}$ hydrochloride (2–5 Ci/mmol) and $^{133}\text{BaCl}_2$ (1–10 mCi/mg) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals, AnalaR or laboratory-grade reagents, were obtained from BDH Chemicals, Poole, Dorset, U.K., or Sigma.

Biosynthetic radiolabelling of avian tracheal mucus with [^{35}S]sulphate and D-[^3H]glucosamine as precursors

Birds were killed by intravenous injection (60 mg/kg body wt. via a wing vein) of sodium pentobarbitone (Sagatal; May and Baker, Dagenham,

Essex, U.K.). The trachea was exposed, and its cranial and caudal ends were cannulated with flanged glass cannulae, dissected free of connective tissue and then suspended in a vertical glass organ bath filled with avian physiological buffer maintained at 41°C and with O₂/CO₂ (19:1) being bubbled continuously. The avian physiological buffer contained 137 mM-NaCl, 2.68 mM-KCl, 0.105 mM-MgCl₂, 1.8 mM-CaCl₂, 11.6 mM-NaHCO₃, 0.4 mM-NaH₂PO₄, 4.4 mM-glucose, gentamicin (50 µg/ml), streptomycin (100 µg/ml), fungizone (2.5 µg/ml) and penicillin (100 units/ml) and pH was maintained at 7.2 by bubbling continuously with O₂/CO₂ (19:1). The tracheal lumen was washed with avian physiological buffer before use. To radiolabel the mucus biosynthetically, the tracheal lumen was filled with buffer solution containing Na₂³⁵SO₄ (10 µCi/ml) and D-[1-³H]-glucosamine hydrochloride (1.25 µCi/ml). Samples of mucus secretion were collected at 10 min intervals over a period of 120 min by flushing out the luminal contents and refilling the lumen with a fresh volume of buffer solution containing radioactively labelled precursors. All samples from a single bird were pooled and kept at 4°C in the presence of 0.02% NaN₃ until further use.

Secretion samples that showed the presence of dislodged mucosal cells on phase-contrast-microscopic examination were discarded. Samples containing DNA were also excluded from this series of experiments. No erythrocytes or polymorphonuclear leucocytes were observed in these secretion samples.

Separation and initial handling of different types of mucus material

Visual inspection of secretion samples revealed that these consisted of materials that varied in their consistency and macroscopic appearance. Thread-like fibrillar material was gently picked out with the aid of a Pasteur pipette and was designated 'fibrillar mucus'. From the rest of the sample, the liquid phase was aspirated and designated 'soluble-phase mucus'. The remaining material, which consisted of viscid gelatinous balls, was designated 'gelatinous mucus'.

Gel filtration

Gel filtration was performed on Ultrogel AcA-22, Bio-Gel A-15m and a variety of Bio-Gel A-type resins. Columns (1.25 cm × 86 cm) were equilibrated and eluted with the appropriate buffers, depending on the nature of the experiments. Fractions of volume 2.5 ml were collected at a flow rate of 2.5 ml/h.

Ion-exchange chromatography

Ion-exchange chromatography was performed on DEAE-cellulose (DE-52). The resin was equili-

brated with the appropriate elution buffers in accordance with the manufacturer's recommendations and the columns (1.5 cm × 8.0 cm) were prepared. After application of the samples, the columns were eluted with 35 ml of the appropriate buffer and then with 150 ml of a linear NaCl gradient (0–0.3 M) made up in the same buffer. Fractions of volume 2.5 ml were collected at a flow rate of 15 ml/h.

Polyacrylamide-gel electrophoresis

Electrophoresis on polyacrylamide gels in the presence and in the absence of sodium dodecyl sulphate and staining of gels were performed by the methods described by Segrest & Jackson (1972), with a range of acrylamide concentrations.

Viscosity measurements

Viscosity of mucus samples was measured with a Ferranti-Shirley cone-and-plate rotational viscometer. The viscosity of mucus samples (0.2 mg/ml) was normally measured over ascending shear rates in the range 9–180 s⁻¹.

$$\text{Viscosity} = \frac{\text{shear stress (dyn/cm}^2\text{)}}{\text{shear rate (s}^{-1}\text{)}} \times 10^{-2}$$

The viscometer was calibrated at 22°C with aqueous solutions of sodium CM-cellulose, which exhibited non-Newtonian properties (Cooke *et al.*, 1978).

Radioactivity measurements

Radioactivity measurements were performed in a Packard PRIAS model liquid-scintillation counter, a Beckman LS 7500 counter and a Nuclear Enterprises 8312 counter, with NE 260 (Nuclear Enterprises, Edinburgh, Scotland, U.K.) as scintillant. Whenever necessary, the counting data were transformed into d.p.m./mg or d.p.m./ml of sample. Calibration curves for counting efficiency of dual radiolabels and for quenching effects were made and values were normalized accordingly.

Chemical determinations

DNA was measured by the diphenylamine method of Burton (1956), with calf thymus DNA (Sigma) as a standard. Free *N*-acetylneuraminic acid was determined by the 2-thiobarbituric acid method of Warren (1959), and total free and bound *N*-acetylneuraminic acid were measured by the resorcinol method of Svennerholm (1957). Uronic acid was measured by the modified carbazole method of Bitter & Muir (1962). The sulphate content of the samples was determined by the method of Mende & Whitney (1978). The carbohydrate composition of the samples was analysed after methanolysis by the method of Clamp *et al.* (1971), by g.l.c.

Papain and Pronase digestion

Papain (100 units) was suspended in 1 ml of 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 6.5, containing 5 mM-EDTA and 5 mM-cysteine hydrochloride and warmed at 65°C for 30 min. Activated papain was then added to 1 ml of mucus sample (1.5 mg/ml), in the same buffer but also containing 2 M-NaCl, and warmed up to 65°C. With insolubilized papain, 10 units of enzyme were added to 100 µg of mucus sample. The mixture was incubated at 65°C at pH 6.5 for 16–24 h.

Pronase (50 units) suspended separately in 1.0 ml of 0.1 M-sodium tetraborate/boric acid buffer, pH 6.8, containing 1 M-KCl was mixed with 1.0 ml of mucus sample (1.5 mg/ml) made up in the same buffer. With insolubilized Pronase, 10 units of enzyme were added to 100 µg of mucus sample. Mixtures were incubated at 37°C for 24 h.

One unit of papain or Pronase is defined as the enzyme activity capable of hydrolysing 1.0 µmol of α -N-benzoyl-L-arginine ethyl ester/min under the above conditions.

Results and discussion

Viscosity properties

The viscosity decreased in the order fibrillar > gelatinous > soluble-phase mucus (Table 1). Dialysis, freeze-drying and reconstitution, although destroying the macroscopic appearance, did not significantly affect the viscosity of fibrillar and gelatinous mucus. Since freeze-drying removes the water by sublimation, it has been pointed out by previous workers (Meyer & Silberberg, 1978) that this treatment should leave the macromolecular structure fairly intact, and hence when water is

restored during the reconstitution step the original structure may well be recovered to some extent. Gentle manual homogenization with a Teflon plunger in a glass tube destroyed the macroscopic appearance of fibrillar and gelatinous mucus without significantly decreasing their viscosities. However, homogenization with a Waring blender, sonication and chemical solubilization by 8 M-urea in the presence and in the absence of 0.1 M-dithiothreitol significantly decreased the viscosity of all mucus samples ($P < 0.001$). Avian tracheal mucus, like other 'gel-phase'-type glycoproteins, responded to urea and reducing agents by showing a significant decrease in its viscosity (Havez *et al.*, 1967; Roberts, 1974, 1976; Boat *et al.*, 1976).

Partial purification of tracheal mucins by gel filtration

The dual-radiolabelled fibrillar, gelatinous and soluble-phase mucins were eluted as single components in the excluded volumes of the Bio-Gel A-15m columns (Fig. 1). Although it has been reported previously that gel filtration of viscous glycoproteins gave poor separation because of flow irregularities and incomplete recoveries (Bhushana Rao *et al.*, 1973), no such difficulty was experienced in the present work. The specific-viscosity values of the mucus samples (1 mg/ml) applied to these columns were in the range 15–60.

Gel filtration on Bio-Gel A-5m, A-50m and A-150m and on Ultrogel AcA-22 in buffer systems such as (a) 0.1 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.5, containing 1 M-NaCl, 5 mM-EDTA and 5 mM-cysteine hydrochloride, (b) 0.1 M-sodium tetraborate/boric acid, pH 6.8, containing 1 M-KCl, (c) 0.1 M-sodium acetate/acetic acid, pH 5.0, and (d)

Table 1. *Effect of physical and chemical solubilization procedures on the viscosity of avian tracheal mucus*

The viscosity was measured of tracheal mucus samples separated from whole secretions either in their native forms or after various treatments listed below. The viscosity of samples (0.2 mg/ml) was measured at shear rate 20 s^{-1} by using a Ferranti-Shirley viscometer. The values are expressed as means $\pm$ S.E.M. for samples collected from seven animals. On each sample three independent measurements were taken.

	Fibrillar mucus	Gelatinous mucus	Soluble-phase mucus
Native samples	47.6 $\pm$ 4.5	32.0 $\pm$ 2.5	8.5 $\pm$ 2.5
Dialysed native samples	44.2 $\pm$ 5.5	28.2 $\pm$ 3.5	6.5 $\pm$ 2.0
Reconstituted mucus samples	42.5 $\pm$ 3.4	26.0 $\pm$ 4.0	6.2 $\pm$ 1.5
Physical homogenization			
(a) Teflon plunger/glass-tube homogenizer; 10 manual strokes	42.5 $\pm$ 2.5	28.5 $\pm$ 3.0	8.0 $\pm$ 3.5
(b) Waring blender homogenizer, 500 rev./ min for 1 min	32.0 $\pm$ 3.0	24.0 $\pm$ 1.5	5.0 $\pm$ 2.5
(c) Sonication for 10 s	12.5 $\pm$ 2.5	12.0 $\pm$ 2.0	4.2 $\pm$ 3.0
Chemical solubilization			
(a) 8 M-Urea	27.0 $\pm$ 3.5	18.0 $\pm$ 4.0	5.0 $\pm$ 3.5
(b) 8 M-Urea + 0.1 M-dithiothreitol	28.0 $\pm$ 3.0	17.5 $\pm$ 3.5	5.0 $\pm$ 3.0

Fig. 1. Bio-Gel A-15m chromatography of dual-radiolabelled mucus separated from avian tracheal secretions *in vitro*. Freeze-dried material was solubilized and dialysed against 0.1 M-Tris/HCl buffer, pH 7.4, and then applied to a Bio-Gel A-15m column (1.25 cm $\times$ 86 cm) equilibrated and eluted with the same buffer. Fractions (2.5 ml) were collected at a flow rate of 2.5 ml/h. All fractions were tested for total hexoses by using the anthrone reagent test, for absorbance at A_{280} and for the presence of ^{35}S and ^3H radioactivities. Recoveries of both radiolabels were over 96%. Fractions (12–20) showing the presence of dual radioactivities were also positive to the anthrone reagent test. $\blacktriangle$, $\bullet$ and $\blacksquare$, ^{35}S radioactivity; $\triangle$, $\circ$ and $\square$, ^3H radioactivity; $\cdots$, A_{280} . (a) Fibrillar mucus; (b) gelatinous mucus; (c) soluble-phase mucus.

0.1M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.5, containing 8 M-urea and 0.1 M-dithiothreitol showed no further resolution and fractionation of these mucins. These mucins, whether or not chemically denatured and reduced, did not permeate polyacrylamide gels made up to acrylamide ranging from 5% to 12.5% (w/v) in the presence or in the absence of sodium dodecyl sulphate and dithiothreitol. Only in gels containing

2.5% (w/v) acrylamide, a faint periodate–Schiff staining and the ^{35}S and ^3H radioactivities were found to be present near the origin of the gels. The anomalous behaviour of glycoproteins on polyacrylamide-gel electrophoresis because of their polydisperse nature (Holden *et al.*, 1971) has been well documented (Bretscher, 1971; Segrest *et al.*, 1971).

Macromolecular aspects of partially purified mucins

Effect of hydrogen-bond-breaking agents and thiol reagents. Treatment with high concentrations of urea or guanidinium chloride in the presence or in the absence of reducing agents had resulted in subfractionation of a variety of glycoproteins on subsequent gel filtration under appropriate chromatographic conditions (Havez *et al.*, 1967; Boat *et al.*, 1976; Roberts, 1976; Allen, 1978; Creeth, 1978). However, incubation of avian tracheal mucins with (a) 50mM-Tris/HCl buffer, pH 8.0, containing 8M-urea, (b) 50mM-Tris/HCl buffer, pH 8.0, containing 8M-urea and 0.1M-dithiothreitol, and (c) 50mM-Tris/HCl buffer, pH 8.0, containing 4M-guanidinium chloride or 0.1M-2-mercaptoethanol for 1h at 37°C produced no difference in their gel-filtration behaviour. This suggests that hydrogen-bond-breaking agents and thiol reagents, which significantly decreased the viscosity, apparently caused no subfractionation and had no effect on the molecular profile of these mucins. Although implication of disulphide bridges in the polymerization of glycoprotein subunits has been reported in a variety of mammalian mucus glycoproteins (Dunstone & Morgan, 1965; Havez *et al.*, 1967; Holden *et al.*, 1971; Doebr & Moghissi, 1973; Roberts, 1976; Marshall & Allen, 1977; Meyer & Silberberg, 1978), it is possible that avian tracheal mucins, like mucins from sheep submaxillary gland (Holden *et al.*, 1971), rat intestine (Forstner *et al.*, 1973) and cat trachea (Kent, 1978), are not made up of subunits, and disulphide bridges are either not involved or they cannot easily be reduced.

Effect of papain and Pronase. Selective action of proteolytic enzymes and their use in the separation

and fractionation of a variety of glycoproteins has been well documented (Slomiany & Meyer, 1972; Snary & Allen, 1972; Allen, 1978; Creeth, 1978). However, papain or Pronase digestion of tracheal mucins before or after their chemical denaturation and reduction did not seem to cause any subfractionation.

This high resistance of avian tracheal mucins to papain and Pronase could be due to their structural characteristics, e.g. (a) the non-glycosylated peptide segments, like the 'naked' region (Masson, 1973), as reported for some mammalian mucins (Donald, 1973; Roberts, 1976; Marshall & Allen, 1977), may not be present, (b) such segments are not being cleaved because of specificity of enzymic actions, (c) the oligosaccharide side chains of the glycosylated segments adjacent to these 'naked' regions perhaps prevented the accessibility of these enzymes, or (d) papain and Pronase may have been rendered ineffective by mucin-enzyme interactions (Scott, 1960; Martin *et al.*, 1969).

Chemical aspects of partially purified tracheal mucins

Analysis of radioactivity data. [³⁵S]Sulphate specific-radioactivity values decreased in the order fibrillar > gelatinous > soluble-phase mucins, whereas ³H specific radioactivity showed a significant decrease in the order gelatinous > fibrillar > soluble-phase mucin (Table 2). Analysis of data on the basis of ³⁵S radioactivity taken as ³⁵SO₄²⁻ and of ³H radioactivity as [³H]glucosamine and not as the metabolically transformed derivatives indicates that the proportion of [³⁵S]sulphate residues compared with that of ³H-labelled residues was lower by many orders of magnitude (Table 2).

Table 2. *Analysis of radiolabelling and the ester sulphate residues content of avian tracheal mucins after repeated filtration on Bio-Gel A-15m*

Tracheal mucus samples, after repeated filtration on Bio-Gel A-15m, were analysed for their ³⁵S- and ³H-radio labelling and total ester sulphate residues content. Calculations of ³H radioactivity in the form of molar contents of [³H]glucosamine were performed for comparative purposes, and these values should not be mistaken as real values, since [³H]glucosamine is normally transformed into other derivatives by metabolic processes. The values given below are expressed on the basis of dry weight of the samples and are means ± S.E.M. for six separate samples pooled from five animals in each case.

	Fibrillar mucin	Gelatinous mucin	Soluble-phase mucin
Analysis on the basis of specific radioactivity			
[³⁵ S]Sulphate (μCi/mg)	2.52 ± 0.5	0.127 ± 0.02	0.004 ± 0.001
³ H label (μCi/mg)	0.51 ± 0.1	0.953 ± 0.05	0.011 ± 0.004
³⁵ S/ ³ H ratio	4.95	0.13	0.35
Analysis on the basis of molar content			
[³⁵ S]Sulphate (pmol/mg)	0.77 ± 0.05	0.04 ± 0.01	0.001 ± 0.001
[³ H]Glucosamine (nmol/mg)	0.21 ± 0.04	0.38 ± 0.06	0.004 ± 0.001
[³⁵ S]Sulphate/[³ H]glucosamine ratio	3.78 × 10 ⁻³	0.098 × 10 ⁻³	0.027 × 10 ⁻³
Total ester sulphate residues (μmol/mg)	0.67 ± 0.07	0.34 ± 0.06	0.03 ± 0.01
[³⁵ S]Sulphate residues/total ester sulphate residues	1.15 × 10 ⁻⁶	0.11 × 10 ⁻⁶	0.03 × 10 ⁻⁶

Total ester sulphate analysis. The total content of ester sulphate residues in these mucins was approx. 10^3 times or more higher than that of [^{35}S]sulphate residues (Table 2), the proportions of fibrillar, gelatinous and soluble-phase mucins being 1:0.5:0.05 (Table 2).

Carbohydrate composition. Although *N*-acetylglucosamine was a major component compared with the other sugar residues present in both fibrillar and gelatinous mucins, the amount of fucose was found to be highest in soluble-phase mucin (Table 3). However, except for fucose, the percentage contents of other sugar residues decreased gradually in the order fibrillar > gelatinous > soluble-phase mucin. The carbohydrate composition of avian tracheal mucins was considerably different from those of some other mammalian mucins (Gallagher & Kent, 1975; Phipps *et al.*, 1977; Allen, 1978; Gibbons, 1978).

However, the presence of xylose in the epithelial mucus glycoproteins, except in secretions from rabbit tracheal explants in organ culture (Gallagher & Kent, 1975), has not been reported previously. The emergence of this peak in the g.l.c. spectrograms was considered to be an artifact as a result of anomalous behaviour of some sulphated sugars. In a preliminary investigation, a commercial preparation of bovine submaxillary-gland mucin (Sigma), which on g.l.c. analysis did not show any peak in the xylose region, was found to give a similar peak after its chemical sulphation (N. Mian, M. Dillon & P. W. Kent, unpublished work). No uronic acid was detected in any of these mucus samples.

Fractionation of avian tracheal mucins by ion-exchange chromatography

Tracheal mucins twice purified by gel-filtration were directly subjected to DEAE-cellulose chromatography. The freeze-dried material (1–2 mg dry wt.) was solubilized in either 10 mM-Tris/HCl

buffer, pH 8.0, or 20 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 10 mM-NaCl, dialysed for 72 h at 4°C against several changes of the appropriate buffers and then subjected to ion-exchange chromatography on DEAE-cellulose. The elution profiles are shown in Fig. 2. All three mucins were found to contain two neutral components, designated B_1 and B_2 , which were eluted in the absence of the NaCl gradient, and a variable number of acid components were eluted from the columns with increasing NaCl concentration. Fibrillar mucin consisted of three acidic components, designated A_1 , A_2 and A_3 , which were eluted at 0.14 M-, 0.19 M- and 0.26 M-NaCl respectively in the linear gradient (Fig. 2a). Gelatinous mucin consisted of acidic components A_1 and A_2 (Fig. 2b) and soluble-phase mucin consisted of acidic component A_1 only (Fig. 2c). The recoveries from the columns were 90–95% with respect to both ^{35}S and ^3H radiolabels, which indicates that hardly any material was lost as a result of adsorption.

Effect of chemical solubilization, reduction and proteolytic digestion on ion-exchange-chromatographic behaviour

Fractionation of tracheal mucins from both mammalian and avian sources by ion-exchange chromatography had shown a considerable variation in the number and type of glycoprotein components being resolved, depending on the experimental conditions, such as urea treatment or papain digestion of the original samples (Gallagher *et al.*, 1975; Gallagher & Kent, 1975; Phipps *et al.*, 1977; Ellis, 1977; Kent, 1978).

Treatment of avian tracheal mucins with 8 M-urea in the presence or in the absence of 0.1 M-dithiothreitol as well as digestion with papain and Pronase before their application to DEAE-cellulose columns equilibrated and eluted with appropriate buffers

Table 3. *Carbohydrate analysis of avian tracheal mucins after repeated filtration on Bio-Gel A-15m*
Tracheal mucins, after repeated filtration on Bio-Gel A-15m, were analysed for their *N*-acetylneuraminic acid residues content chemically and for the rest of their carbohydrates by g.l.c. The values are expressed as percentages of the dry weight of the samples and are the average of two g.l.c. determinations on three separate samples collected from six animals. Molar ratios of monosaccharides were calculated by taking *N*-acetylglucosamine as 1.0.

	Fibrillar mucin		Gelatinous mucin		Soluble-phase mucin	
	(%)	(molar ratio)	(%)	(molar ratio)	(%)	(molar ratio)
Fucose	9.0	0.59	8.2	0.71	9.8	1.50
Galactose	11.8	0.70	9.2	0.72	8.1	1.12
<i>N</i> -Acetylgalactosamine	13.0	0.63	8.8	0.57	4.4	0.50
<i>N</i> -Acetylglucosamine	20.5	1.00	15.5	1.00	8.8	1.00
Mannose	5.4	0.32	4.5	0.36	1.8	0.25
Glucose	4.5	0.27	2.7	0.21	1.8	0.25
<i>N</i> -Acetylneuraminic acid	4.3	0.15	4.0	0.19	2.2	0.18

Fig. 2. DEAE-cellulose chromatography of Bio-Gel-fractionated avian mucus

Mucus samples after repeated filtration through Bio-Gel A-15m were extensively dialysed against distilled water and then freeze-dried. The resulting materials were then dialysed against 20mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 7.2, containing 10mM-NaCl and applied to DE-52 DEAE-cellulose columns (1.5 cm $\times$ 8.0cm) equilibrated with the same buffer, followed by 150ml of linear gradient (0–0.3 M) of NaCl in the same buffer. Fractions (2.5 ml) were collected at a flow rate of 15 ml/h. $\blacktriangle$, $\bullet$ and $\blacksquare$, ^{35}S radioactivity; $\triangle$, $\circ$ and $\square$, ^3H radioactivity; , [NaCl], measured by conductivity. Recoveries of ^{35}S and ^3H radioactivities and of actual amount of material were approx. 98% for fibrillar mucus (a), 96% for gelatinous mucus (b) and 95% for soluble-phase mucus (c). The proportions of total amounts of glycoproteins determined on the basis of their dry weights were 4:2:1:1:2 for components B_1 , B_2 , A_1 , A_2 and A_3 of fibrillar mucus (a), 4:3:1:2 for components B_1 , B_2 , A_1 and A_2 of gelatinous mucus (b) and 4:3:3 for components B_1 , B_2 and A_1 of soluble-phase mucus (c).

showed no difference in the ion-exchange-chromatographic behaviour or in the net and relative ^{35}S and ^3H radioactivities of any of the neutral and acidic components of the treated samples compared with those of untreated samples.

Chemical aspects of fractionated glycoprotein components of tracheal mucins

Although components B_1 and B_2 from all three types of mucin possess both ^{35}S and ^3H radiolabels, the total amounts of these radiolabels in these components are usually lower than those of acidic components. Although $^{35}\text{S}/^3\text{H}$ ratios for components B_1 and B_2 are more consistent for fibrillar and soluble-phase mucins, these ratios for acidic components from both fibrillar and gelatinous mucins show considerable variation. The $^{35}\text{S}/^3\text{H}$ ratio of acidic components of fibrillar mucin (Table 4) gives the impression that [^{35}S]sulphate labelling was considerably higher than ^3H labelling; analysis of the data on the basis of molar amounts of the two residues indicated that all components contained substantially higher amounts of ^3H label (> 0.1 nmol) than of [^{35}S]sulphate (< 1.0 pmol) (Table 4).

Chemical analysis of glycoprotein components

Both neutral components B_1 and B_2 of fibrillar, gelatinous and soluble-phase mucins were similar in

their carbohydrate composition (Table 5). Although components A_1 of both fibrillar and gelatinous mucins showed a close resemblance in their carbohydrate composition, component A_1 of soluble-phase mucin was different. The sugar contents of acidic components A_2 of both fibrillar and gelatinous mucins and component A_3 of fibrillar mucin were similar. The molar proportions of the four principal sugars, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and galactose, were 1:0.66:0.62:0.66. The neutral components contained extremely small amounts of *N*-acetylneuraminic acid and ester sulphate (Tables 5 and 6). The acidic components of fibrillar and gelatinous mucins had considerably high ester sulphate contents, and there was a close resemblance in the ester sulphate and *N*-acetylneuraminic acid contents of the corresponding components of fibrillar and gelatinous mucins (Tables 5 and 6).

Concluding remarks

The tracheal preparation technique *in vitro* offers a unique opportunity to obtain different types of avian tracheal epithelial mucins. Partially purified dual-radiolabelled mucins, namely fibrillar, gelatinous and soluble phase, were polydisperse aggregates of high molecular weight that did not seem to comprise subunit structures held together

Table 4. Analysis of radiolabelling of glycoprotein components of avian tracheal mucins separated by ion-exchange chromatography

Tracheal mucins after repeated filtration on Bio-Gel A-15m were dialysed and freeze-dried. The freeze-dried material (approx. 5 mg) was solubilized in 0.5 ml of appropriate buffer solution and subjected to chromatography on DEAE-cellulose as described in the text. Peaks of dual-radiolabelled material were separately pooled and extensively dialysed. Total radioactivities of each component were calculated and are expressed as $\mu\text{Ci}/\text{mg}$ of the glycoprotein component. Calculations of ^3H radioactivity in the form of molar content of [^3H]glucosamine were based on the assumption stated in Table 2. The values given are the averages from three separate fractionation experiments. Acidic component A_3 and acidic components A_2 and A_3 were not present in gelatinous and soluble-phase mucins respectively.

	Specific radioactivity			Molar contents	
	[^{35}S]Sulphate ($\mu\text{Ci}/\text{mg}$)	^3H ($\mu\text{Ci}/\text{mg}$)	$^{35}\text{S}/^3\text{H}$ ratio	[^3H]Glucosamine (nmol/mg)	[^{35}S]Sulphate (pmol/mg)
Fibrillar mucin					
Neutral components B_1	0.04	0.05	0.80	0.020	0.0118
B_2	0.08	0.10	0.80	0.040	0.0237
Acidic components A_1	1.60	0.22	7.29	0.088	0.4752
A_2	0.48	0.08	6.02	0.032	0.1425
A_3	0.28	0.06	4.67	0.024	0.0831
Gelatinous mucin					
Neutral components B_1	0.004	0.20	0.02	0.080	0.0012
B_2	0.025	0.10	0.25	0.040	0.0074
Acidic components A_1	0.058	0.58	0.10	0.232	0.0172
A_2	0.036	0.05	0.72	0.020	0.0106
Soluble-phase mucin					
Neutral components B_1	0.0020	0.03	0.060	0.012	0.0006
B_2	0.0005	0.01	0.050	0.004	0.0001
Acidic component A_1	0.0015	0.06	0.025	0.024	0.0004

Table 5. *Carbohydrate analysis of glycoprotein components of avian tracheal mucins separated by ion-exchange chromatography*

Glycoprotein components separated from tracheal mucin as described in Table 4 were subjected to carbohydrate analysis as described in Table 3. The values are expressed as percentages of the dry weight of the sample and are the average of results obtained by the analysis of three separate samples.

	<i>N</i> -Acetyl-glucosamine	<i>N</i> -Acetyl-galactosamine	Fucose	Galactose	Glucose	Mannose	<i>N</i> -Acetyl-neuraminic acid
Fibrillar mucin							
Neutral components B ₁	2.2	1.1	2.5	1.8	—	—	0.06
B ₂	2.2	0.8	2.5	1.6	—	—	0.21
Acidic components A ₁	5.3	3.5	2.5	2.8	0.9	2.7	3.15
A ₂	5.3	3.5	0.8	2.7	1.8	1.8	0.43
A ₃	5.5	4.0	0.8	2.8	1.8	0.9	0.24
Gelatinous mucin							
Neutral components B ₁	2.2	0.8	2.5	1.8	—	—	0.09
B ₂	2.4	1.1	2.5	1.8	—	—	0.18
Acidic components A ₁	5.3	3.3	2.5	2.8	0.9	2.7	3.24
A ₂	5.5	3.5	0.8	2.7	1.8	1.8	0.43
Soluble-phase mucin							
Neutral components B ₁	2.2	1.1	2.5	1.8	—	—	0.12
B ₂	2.2	1.1	2.5	1.8	—	—	0.18
Acidic component A ₁	4.4	2.2	4.9	2.7	0.9	1.8	2.10

Table 6. *Total ester sulphate residue content of glycoprotein components of avian tracheal mucins*

Total ester sulphate residues content was analysed on glycoprotein components of avian tracheal mucins. The values are expressed as percentages of the dry weight of the sample and are averages of values obtained from three separate fractionation experiments. Other details are as given in Table 4.

Glycoprotein components	Ester sulphate residues content (%)		
	Fibrillar mucin	Gelatinous mucin	Soluble-phase mucin
Neutral components B ₁	0.03	0.03	<0.01
B ₂	0.06	0.06	<0.01
Acidic components A ₁	1.2	1.0	0.3
A ₂	1.8	2.1	Absent
A ₃	3.1	Absent	Absent

either by disulphide bridges or by papain- or Pronase-cleavable peptide bonds. Although these mucins behaved as single components on gel fractionation, a high degree of internal inhomogeneity nevertheless persisted. They varied considerably in their extent of ³⁵S and ³H radiolabelling, in chemical analysis and in the number of their constituent glycoproteins. Overall physical properties of these mucins appeared to be correlated with the chemical structure of their glycoprotein components. Furthermore, the remarkable similarity between the corresponding components of fibrillar and gelatinous mucins with respect to their charge density and distribution, carbohydrate composition and ester sulphate contents suggested that the difference between the two insoluble mucins was due to the presence of an additional acidic component A₃ in the fibrillar mucin. Although none of these glycoprotein components was exclusively sialylated or sulphated, none of them contained uronic acid.

Although these acidic glycoproteins were constituents of the secreted mucins, and their synthesis and packaging within the tracheal luminal cells was not directly monitored, the data on their chemical analysis and radiolabelling aspects pointed out some interesting features. Although the total contents of constituent sugar residues were similar in different acidic glycoproteins, the total ester sulphate contents decreased in the order A₃ > A₂ > A₁, and the amounts of [³⁵S]sulphate and ³H label varied in the order A₃ < A₂ < A₁. Such results could be explained by suggesting that, in addition to the accepted processes involved in the stepwise sulphation of glycoproteins, there is an alternative process that utilizes an existing pool of free oligosaccharides or related oligomeric precursors of varied degrees of sulphation. In this manner, the glycoprotein components made from the most sulphated oligosaccharides would have the least available sites for the incoming [³⁵S]sulphate residues. Similarly, this

alternative process would explain the relatively consistent amount of total sugar residues as well as of *N*-acetylglucosamine in these acidic glycoprotein components while allowing for variation in the extent of ^3H radiolabel.

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