Structure and Metabolism of Glycoproteins and Glycosaminoglycans Secreted by Organ Cultures of Rabbit Trachea

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(Received 28 October 1974)

1. When cultured in medium 199 in an atmosphere of $O_2 + CO_2$ (95:5) rabbit tracheal explants retained their viability for up to 21 days. 2. The explants secreted into the culture medium three electrophoretically separable components which were eluted in the non-retarded fraction from Sephadex G-200. 3. Digestion with papain and fractionation with a LiCl gradient on DEAE-cellulose resulted in the separation of these components, which were identified as a sialic acid-rich glycoprotein of epithelial origin, and chondroitin sulphate and hyaluronic acid from sub-epithelial cartilage and connective tissue. 4. Incorporation of radioactive precursors (D-[U- ^{14}C]glucose, D-[1- ^{14}C]glucosamine, D-[1- ^{14}C]galactose and $Na_2^{35}SO_4$) into these secreted macromolecules was indicative of their active biosynthesis by the tracheal tissue.

The tracheal epithelium is involved in a number of pathological states (Yeager, 1971) and, in consequence, tracheal organ cultures have been used mainly for studies of this specialized cellular region. For example, organ cultures of embryonic human and calf trachea (Hoorn, 1966) were used to examine the interaction of respiratory viruses with target epithelial cells. Histochemical and radioautographic studies on cultures of the tracheal mucosa have indicated the possible existence of several neutral and acidic glycoproteins (Lamb & Reid, 1969) which undergo quantitative and perhaps qualitative changes in the hypersecretory states such as are found in chronic bronchitis and cystic fibrosis (Lev & Spicer, 1965; Reid, 1970).

Earlier work on the metabolism of tracheal glycoproteins involved measurement of the incorporation of radioactive precursors into macromolecular products, but little information was obtained on the source and structure of these radioactive components (Kent *et al.*, 1969, 1971; Formijne *et al.*, 1964; Yeager *et al.*, 1971). Ellis & Stahl (1973*a,b*) published detailed findings on the glycoproteins secreted by canine tracheal cultures, from which they isolated a glycoprotein fraction, AG1, which consisted of 50% carbohydrate, with galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid as the main monosaccharide constituents. This fraction represented secretions from both mucous glands and goblet cells, with the latter contributing the major portion of sulphated glycoproteins.

The trachea, however, is a complex structure in which the epithelial mucosa is borne on a layer of sub-mucosal connective tissue supported by rings of hya-

line cartilage embedded in a network of collagen and elastic fibres. Under organ-culture conditions non-epithelial regions can be expected to contribute to the total secretory activity of the system, particularly since cartilage proteoglycans have been detected in the culture fluids from rat costal cartilage (Wasteson *et al.*, 1972). In bovine tracheal cartilage, about 20% of its dry weight is in the form of proteoglycans which are present as a heterogeneous population of macromolecules with considerable variation in protein: polysaccharide ratios (Heinegard, 1972).

In view of these findings, the present work was designed to study the secretory activity of both epithelial and connective-tissue regions of rabbit trachea in organ culture. A preliminary report has been published (Gallagher & Kent, 1973).

Materials and Methods

Materials

Medium 199, containing Earle's salts, was obtained from Burroughs Wellcome and Co., Beckenham, Kent, U.K. Penicillin and streptomycin, prepared in a single formulation called 'Crystamycin', was supplied by Glaxo Laboratories Ltd., Greenford, Middx., U.K., and Fungizone (amphotericin B) was obtained from E. R. Squibb and Sons, New York, N.Y., U.S.A. D-[1- ^{14}C]Glucosamine (2–4 mCi/mmol), D-[U- ^{14}C]glucose (196 mCi/mmol), D-[1- ^{14}C]galactose (2–4 mCi/mmol) and $Na_2^{35}SO_4$ (up to 100 mCi/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Crystalline papain was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., and

hyaluronidase (4000 U.S.P. units/mg) from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Nembutal was supplied by Mawson and Proctor, Newcastle-upon-Tyne, U.K.

Culture medium

Explants were maintained in Medium 199 containing 0.22% NaHCO_3 with 100 units of penicillin/ml, 100 μg of streptomycin/ml and 2.5 μg of Fungizone/ml added to control microbial growth.

Organ cultures of rabbit trachea

Rabbits (2.5 kg) of either sex were killed by Nembutal injection, the trachea was exposed by dissection, and haemostats were clamped in position at both ends of the trachea, which was then cut away from the bronchi and larynx. The outer surface was briefly wiped with cotton wool (moistened with 70% ethanol) and the trachea then washed in two changes of warm (37°C) culture medium before those segments of tissue damaged by the haemostats were cut away. After two further washes in warm medium the

trachea was cut into small segments (0.5 cm²–1 cm²) and each segment placed on the incised surface of a plastic Petri dish, two segments per dish (Hoorn, 1966). Culture medium (5 ml) was added to each dish and incubation at 37°C in an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5) was continued for 7–9 days, although in some instances cultures were maintained for up to 21 days. The culture medium was changed at 1–2-day intervals. On some occasions radioisotopes were added to the culture medium at the following concentrations: D-[1-¹⁴C]glucosamine, 0.05 $\mu\text{Ci/ml}$; D-[1-¹⁴C]galactose, 0.05 $\mu\text{Ci/ml}$; D-[U-¹⁴C]glucose, 0.4 $\mu\text{Ci/ml}$; and $\text{Na}_2^{35}\text{SO}_4$, 1.5 $\mu\text{Ci/ml}$.

Collection and fractionation of tracheal secretions

After 24–48 h in culture, small amounts of insoluble mucous gel were seen floating in the culture medium and adhering to the epithelial surface of the explants. Spent medium and gel were removed from the cultures with a Pasteur pipette. The gel was separated by centrifugation and, after several washings with water, was stored in the deep-freeze.

Scheme 1. Fractionation and characterization of tracheal secretions

The spent medium containing 'soluble secretions' was dialysed exhaustively against water containing NaN_3 (0.02%, w/v), concentrated 8–10-fold by negative pressure dialysis, and then stored at 4°C. In some experiments deep-freezing gave rise to precipitate of glycoprotein which did not redissolve on thawing. The concentrated secretions were then examined by electrophoresis, ion-exchange chromatography, gel filtration and chemical analysis according to the outline given in Scheme 1; in some cases the native secretions were first digested with papain to facilitate their characterization.

Electrophoresis

Secretions and column fractions were examined as a routine by zone electrophoresis on cellulose acetate strips in a Shandon horizontal electrophoresis apparatus containing 0.07M-barbitone buffer, pH 8.6. Buffer-soaked strips, with excess of moisture removed by gentle blotting, were placed in the apparatus and up to 25 μl of solution was applied in a thin line before a potential of 12–15 V/cm was applied for 40–60 min. Strips were stained with Alcian Blue (1%, w/v) in acetic acid (2.5%, v/v) or with periodate–Schiff reagent to detect glycoproteins and glycosaminoglycans. Protein-containing substances were located with Nigrosine (0.002%, w/v, in water).

Gel filtration

Sephadex G-200. The gel was allowed to swell for 3 days at room temperature in an excess of 0.05M-Tris-HCl buffer, pH 8.0, containing 0.02% (w/v) NaN_3 , and fines were removed by repeated decantation. The slurry was packed into a column (40 cm $\times$ 3.2 cm) and equilibrated with several bed volumes of buffer before use. Samples of tracheal secretions were applied in a maximum volume of 5 ml and eluted by upward flow at a flow rate of 12 ml/h.

Sephacrose 2B. The pre-swollen gel, suspended in an excess of 0.15M-NaCl containing 0.02% NaN_3 , was used to prepare a column of final bed size 36 cm $\times$ 2 cm. Tracheal secretions were applied in volumes of up to 4 ml and eluted by downward flow at a rate of 5 ml/h.

Sephadex G-25. The gel was equilibrated for several hours in an excess of 1.0M-NaCl. A column (38 cm $\times$ 1 cm) was prepared and equilibrated further with NaCl before fractionation of 1 ml samples of chondroitin sulphate at a flow rate of 10 ml/h.

Ion-exchange chromatography

This technique was carried out by using both DEAE-cellulose DE-52 and DEAE-Sephadex A-25. DEAE-Sephadex A-25 was used essentially as described by Schmidt (1962); the exchanger was equilibrated with 0.1M-NaCl, packed into a column

(10 cm $\times$ 1 cm) and samples were applied to the column in 0.1M-NaCl. Stepwise elution was performed by passing through 3 bed volumes each of 0.25M-, 0.5M-, 1.25M- and 2.0M-NaCl, with the latter two solutions made in 0.01M-HCl. The flow rate was 20 ml/h.

DEAE-cellulose DE-52 was equilibrated with 0.01M-LiCl in 0.05M-acetate buffer (pH 4.4) with fines removed as recommended by the manufacturers. A column (1.5 cm $\times$ 5 cm) was prepared and samples were applied in starting buffer before elution with a linear gradient of LiCl from 0.01 to 1.0M in acetate buffer (Hallen, 1972).

The effluent from gel-filtration and ion-exchange columns was usually monitored at 254 nm with an LKB Uvicord absorptiometer; the volumes of fractions collected from columns are given in the Figure legends. Column peaks were dialysed and concentrated before chemical analysis or electrophoresis.

Papain digestion

Crystalline papain (0.5 mg/ml) was added to 0.1M-citrate buffer, pH 5.6, containing 1.0M-NaCl, 5 mM-EDTA and 1 mM-cysteine. The enzyme was activated by being heated at 60°C for 30 min (Scott, 1960). Freeze-dried samples of tracheal secretions (1–5 mg/ml) were suspended in this solution and incubated at 60°C for 4 h. After cooling, protein was precipitated by addition of 90% (w/v) trichloroacetic acid to a final concentration of 5% (w/v). The mixture was left at 4°C for 2 h and the precipitate separated by centrifugation. After a single extraction with cold 5% (w/v) trichloroacetic acid, the precipitate was discarded and the supernatants were pooled, dialysed exhaustively against water and then concentrated by either rotary evaporation or freeze-drying.

Isolation of glycosaminoglycans with cetylpyridinium chloride

Papain-digested material was shaken overnight at room temperature with 0.5M-NaOH in the presence of NaBH_4 (0.05%, w/v). After neutralization with 1M-HCl, glycosaminoglycans were precipitated by addition of 3 vol. of ethanol and a few drops of 3M-NaCl. The precipitate formed after standing at 4°C for 2 h was separated by centrifugation, washed with ethanol and air-dried. The precipitate was then dissolved in water and glycosaminoglycans were re-precipitated by addition of 1% (w/v) cetylpyridinium chloride (in 0.005M- Na_2SO_4) to a final concentration of 0.1% cetylpyridinium chloride (Scott, 1960). The precipitate was collected after standing overnight at room temperature, extracted several times at 37°C with 2–3 ml of ethanol saturated with sodium acetate to remove the cetylpyridinium chloride and, after a final washing with ethanol, the precipitate was air-dried.

Hyaluronidase digestion

Freeze-dried preparations of tracheal secretions were dissolved in 0.05M-phosphate-citrate buffer (pH 5.5) in 0.1M-NaCl and testicular hyaluronidase was added to give a concentration of 0.25mg of enzyme/ml (Mathews & Inouye, 1961). Samples were incubated in stoppered tubes at 40°C for 16h and then either freeze-dried before fractionation on Sephadex G-25 or dialysed against water and electrophoresed.

Analytical methods

Uronic acid was measured by the Bitter & Muir (1962) modification of the carbazole reaction, with glucuronic acid and hyaluronic acid as standards. Hexosamines were measured by the Elson-Morgan reaction as recommended by Boas (1953), with glucosamine hydrochloride as standard. Samples were hydrolysed with 4M-HCl in sealed tubes under N₂ for 8h at 100°C, hydrolysates dried over NaOH in a vacuum desiccator and redissolved in water before analysis. Sialic acid was determined by the thiobarbituric acid method of Warren (1959) with N-acetylneuraminic acid as standard; glycoproteins were hydrolysed for 1h with 0.05M-H₂SO₄ at 80°C before analysis. The monosaccharide composition of glycoproteins and glycosaminoglycans was determined by the g.l.c. method of Clamp *et al.* (1971) after hydrolysis of dried samples for 24h at 90°C with methanolic 1M-HCl in sealed tubes under N₂. The glucose concentration of the culture medium was measured by the method of Dahlqvist (1960).

Radioactivity determinations

Aqueous samples (maximum volume 0.2ml) were placed in glass or plastic vials containing 7ml of a scintillation mixture made of 8g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] and 80g of naphthalene in 1 litre of toluene-methoxyethanol (3:2, v/v). Radioactivity was measured in a Beckman LS-200B liquid-scintillation spectrometer with the factory set window for ¹⁴C giving a counting efficiency of 85%. On occasions, larger sample volumes (up to 1ml) were counted for radioactivity by mixing with 10ml of Aquasol (New England Nuclear Corp., Dreieichenhain, West Germany).

After electrophoresis of tracheal secretions the distribution of radioactivity along cellulose acetate strips was determined by cutting the strip into 0.5cm-wide segments and counting the radioactivity of each segment in 7ml of the toluene-methoxyethanol scintillant.

Results

Under the culture conditions described above, histology of explants of rabbit trachea (kindly carried out by Dr. M. Fillenz) showed little signs of gross morphological changes at least during the first 7 days in culture. Cultures were rarely kept for longer periods of time, although it is noteworthy that glucose utilization was maintained at a near-constant rate of 85 µg/24h per mg dry wt. of tissue for up to 14 days, and that the ciliary activity of the epithelial layer persisted for at least 3 weeks. The radioisotope incorporation into tracheal secretions and the type and quantity of these secretions showed little alteration during 7–10 days in culture.

Incorporation of radioactive precursors into soluble extracellular macromolecules

When tracheal explants were incubated for 48h with D-[1-¹⁴C]glucosamine, 8.50% of the added radioactivity was incorporated into non-diffusible materials; D-[1-¹⁴C]galactose (6.25% incorporation), D-[U-¹⁴C]glucose (7.10% incorporation) and Na₂³⁵SO₄ (0.22% incorporation) were also detected in non-diffusible material after a 48h incubation. During incubation of the explants with D-[1-¹⁴C]glucosamine for 10 days with changes of medium every 48h the radioactivity incorporated increased from 8.5% in the first 48h to a steadily maintained value of 10–11%, the increase presumably occurring as a result of the time required for isotopic equilibration of extracellular D-[1-¹⁴C]glucosamine with intracellular metabolic pools of amino sugar derivatives.

Electrophoresis of the concentrated secretions showed three Alcian Blue-staining bands designated A, B and C in order of their electrophoretic mobilities (Fig. 1); only component C stained with periodate-Schiff reagent. Components A and B corresponded in mobility to chondroitin sulphate and hyaluronic acid respectively. After incubation of tracheal explants with the radioactive precursors described previously, electrophoresis, followed by measurement of the distribution of radioactivity along the cellulose acetate strip, showed that all three secreted components incorporated D-[1-¹⁴C]glucosamine and D-[1-¹⁴C]galactose, with Na₂³⁵SO₄ confined mainly to components A and C (Fig. 1). D-[U-¹⁴C]Glucose was also incorporated into all three components, but the major portion of the radioactivity was found in component C. In all cases some radioactivity remained at the origin.

Papain-digested secretions gave a similar electrophoretic pattern, except that component C had a slightly increased mobility and component A moved as a tighter band.

Fig. 1. Cellulose acetate electrophoresis of rabbit tracheal secretions labelled with D-[1-¹⁴C]glucosamine, D-[1-¹⁴C]galactose, D-[U-¹⁴C]glucose and Na₂³⁵SO₄.

Cultures were incubated for 48 h with each radioactive precursor and the concentrated secretions electrophoresed after dialysis to remove non-incorporated radioisotope. The cellulose acetate paper was cut into 0.5 cm segments for radioactivity determination. The graph shows the distribution of radioactivity along the cellulose acetate strip of D-[1-¹⁴C]glucosamine-labelled secretions (—) and the effect of hyaluronidase digestion on the amount of radioactivity in each secreted component (---). The lower part of the Figure shows the relative incorporation of all radioisotopes into the tracheal secretions.

Analysis of monosaccharides in tracheal secretions

The monosaccharides found in the culture fluid are given in Table 1; it is notable that monosaccharide concentrations showed in general only small fluctuations during the 9 days culture period used in the experiment. When the epithelium was scraped away from the explants with a scalpel blade and pressure applied to the damaged epithelial surface with the flat face of the blade to damage the gland cells, no insoluble mucus was found, and the tracheal secretions contained significantly less fucose (sixfold decrease) and sialic acid (fourfold decrease), although uronic acid contents were unaffected (Table 2). Electrophoresis showed that epithelial removal resulted in the loss of component C from the secretions. Component C therefore appeared to be a sialic acid-containing glycoprotein of epithelial origin, whereas components A and B were derived from tracheal cartilage and connective tissue. The uronic acid concentrations found in the secretions were indicative of a high glycosaminoglycan content.

Fractionation of tracheal secretions

Gel filtration. When passed through a Sephadex G-200 column, the concentrated secretions, radioactively labelled with either D-[U-¹⁴C]glucose or D-[1-¹⁴C]glucosamine, gave a single major peak

Table 1. Secretion of non-diffusible (i.e. bound) monosaccharides during 9 days in culture

Duplicate cultures were used in this experiment and each culture contained 17 mg dry wt. of tracheal tissue. The spent medium from these cultures was pooled, dialysed and freeze-dried before analysis by g.l.c. Medium was renewed every 48 h. Electrophoresis of the spent medium failed to show the presence of serum components, except in the discarded medium from the first 24 h of incubation, and thereafter medium was renewed at 48 h intervals.

	Secretion (μ mol/48 h per g dry wt.)
Fucose	1.38 $\pm$ 0.76
Xylose	2.80 $\pm$ 0.59
Uronic acid	22.0 $\pm$ 4.7
Mannose	2.5 $\pm$ 0.74
Galactose	9.0 $\pm$ 1.9
N-Acetylgalactosamine	27 $\pm$ 5.4
N-Acetylglucosamine	12 $\pm$ 2.5
Sialic acid	3.1 $\pm$ 0.47

eluted in the void volume of the column (Fig. 2). Electrophoresis showed that all three secreted components were present in this peak with their relative radioactivities unchanged. Papain digestion altered this elution profile (Fig. 2), although the main peak still emerged with the void volume and contained all

Table 2. *G.l.c. analysis comparing the amounts of non-diffusible monosaccharides secreted by explants with and without a functioning epithelium*

After removal of the epithelium, explants were incubated for 48 h and the medium was discarded. Fresh medium was added and the incubation continued for a second 48 h period, after which the medium was dialysed and freeze-dried before analysis. There was no indication of regeneration of ciliary activity during this time. Control cultures, with an intact epithelium, were similarly incubated.

	Molar ratios of sugars present (uronic acid = 1)	
	Whole explants	Explants without epithelium
Fucose	0.13	0.02
Xylose	0.14	0.13
Uronic acid	1.00	1.00
Mannose	0.18	0.11
Galactose	0.61	0.34
<i>N</i> -Acetylgalactosamine	1.50	1.30
<i>N</i> -Acetylglucosamine	0.80	0.54
Sialic acid	0.26	0.07

Fig. 2. *Effect of papain digestion on the fractionation on Sephadex G-200 of tracheal secretions labelled with D-[1-¹⁴C]glucosamine*

Medium from tracheal cultures incubated for 48 h with D-[1-¹⁴C]glucosamine was dialysed, concentrated and fractionated on a Sephadex G-200 column (40 cm × 3.2 cm) (—). The effect of papain digestion on the radioactivity elution profile of a similar sample of incubation medium (----) was assessed after precipitation of the digestion products with trichloroacetic acid and dialysis and concentration of the supernatant as described in the Materials and Methods section. Fractions (5.5 ml) were collected and recoveries of radioactivity were over 94%.

three secreted components on electrophoresis; the other two peaks (fractions 32–37 and 38–50) contained mainly component A, together with small quantities

Fig. 3. *Sepharose 2B chromatography of D-[1-¹⁴C]glucosamine-labelled secretions*

Medium from tracheal cultures incubated for 48 h with D-[1-¹⁴C]glucosamine was dialysed and concentrated before fractionation on a column of Sepharose 2B (36 cm × 2.5 cm); 3.2 ml fractions were collected and pooled as indicated. Only 70% of the added radioactivity was recovered from the column.

of component B as assessed by the weak Alcian Blue staining of this component.

Fractionation on Sepharose 2B resulted in a partial separation of the secretions (Fig. 3); the radioactive peak at the void volume consisted mainly of component B, with a very faint Alcian Blue-staining band in the region of component C. The intermediate fractions (nos. 20–36) contained all three components, although again the staining in the region of component C was very weak. Fractions 36–48, emerging with the total included volume V_1 , contained only components A and B. Of the total bound uronic acid applied to the column 68% was found in the intermediate fractions, 20% in the V_1 peak and 12% in the V_0 peak, giving an overall recovery of 95%. However, only 70% of the applied radioactivity was recovered, and electrophoretic evidence suggested that the column losses were mainly due to poor recoveries of the epithelial glycoprotein.

Ion-exchange chromatography of papain-digested secretions. As shown in Fig. 4, three principal radioactive peaks resulted on fractionating papain-digested secretions with DEAE-cellulose DE-52, electrophoresis indicating that the three electrophoretic components A, B and C were eluted in an order expected from their electrophoretic mobilities (see Fig. 1). In a LiCl gradient, the concentrations at which components A and B emerged from the column corresponded to those of commercial preparations of chondroitin sulphate and hyaluronic acid respectively. Prior digestion of the native secretions with papain was an essential prerequisite to ionexchange fractionation, otherwise as with Sepharose 2B there were considerable losses of the glycoprotein (component C).

Fractionation of papain-digested secretions on DEAE-Sephadex A-25, by the stepwise elution technique advocated by Schmidt (1962) for separating glycosaminoglycans, gave three peaks at 0.25M-, 0.5M- and 1.25M-NaCl corresponding to components C, B and A respectively on electrophoresis, but the reproducibility and column recoveries (75% yield of added radioactivity) were less satisfactory than with DEAE-cellulose.

Analysis of separated fractions

Analysis of the monosaccharides present in components A, B and C isolated from the DEAE-cellulose fractionation showed that uronic acid was absent from component C but was a major constituent of components A and B, in which the uronic acid:hexosamine ratios were close to 1 (Table 3). The prin-

cipal amino sugars were N-acetylgalactosamine in component A and N-acetylglucosamine in component B. Chemical determinations of the uronic acid (carbazole):hexosamine (Elson-Morgan) ratios gave values of 1.1 for component A and 1.15 for component B. The results are in accordance with the identification of component A as chondroitin sulphate and component B as hyaluronic acid. It is noteworthy, however, that total uronic acid and hexosamine concentrations measured by g.l.c. were about 30% lower than those obtained by chemical assays. J. R. Clamp (personal communication) has also noted that glycosaminoglycans give low yields when analysed by g.l.c., presumably because of incomplete hydrolysis of the polysaccharide chains in methanolic HCl. Nevertheless g.l.c. is valuable for glycosaminoglycan analysis because it provides the main amino sugar present and gives an approximate 1:1 uronic acid:hexosamine ratio.

When papain-digested secretions were isolated by cetylpyridinium chloride precipitation electrophoresis showed that components A and B were recovered from the precipitate, and their carbohydrate analyses after DEAE-cellulose ion-exchange separation were essentially similar to those given in Table 3.

Hyaluronidase digestion

Native tracheal secretions, labelled with D-[1-¹⁴C]-glucosamine, were checked for their susceptibility to digestion with testicular hyaluronidase; after incubation, the digest was dialysed, freeze-dried and electrophoresed simultaneously with non-digested material treated in the same way but without the enzyme present. The radioactivity distribution along the electrophoresis strip (Fig. 1) showed that component B (hyaluronic acid) was almost completely degraded into diffusible tetrasaccharides by the enzyme, with little residual radioactivity remaining in the component-B region. The enzyme decreased the radioactivity by 85% in the region of component A, but had little effect on the glycoprotein fraction (component C). Hyaluronidase digestion of the chondroitin sulphate isolated from the secretions by

Fig. 4. DEAE-cellulose DE-52 ion-exchange chromatography of papain-digested tracheal secretions labelled with D-[1-¹⁴C]glucosamine

Papain-digested secretions were equilibrated by dialysis with 0.05M-acetate buffer, pH 4.4, in 0.01M-LiCl before application to the ion-exchange column. Starting buffer (10ml) was passed through the column and the gradient of LiCl (----) from 0.01 to 1.0M was applied in a total volume of 100ml. Fractions (3.2ml) were collected and 98% of the added radioactivity was recovered from the column.

Table 3. Monosaccharide contents and specific radioactivities of tracheal secretions

Explants were incubated with D-[1-¹⁴C]glucosamine (0.05 μ Ci/ml) for 48 h and secretions fractionated, after papain digestion, on DEAE-cellulose DE-52. The separated fractions were analysed for hexosamine by the Elson-Morgan reaction, uronic acid by the carbazole method and sialic acid by the thiobarbituric acid method of Warren (1959). See the Materials and Methods section for details.

	Glycoprotein (component C)	Hyaluronic acid (component B)	Chondroitin sulphate (component A)
Hexosamine (μ mol/g dry wt. of tissue)	8.6	13.2	29.0
Uronic acid (μ mol/g dry wt. of tissue)	—	14.2	32.5
Sialic acid (μ mol/g dry wt. of tissue)	2.8	—	—
Specific radioactivity (c.p.m./ μ mol of hexosamine)	5.4×10^3	3.6×10^3	0.42×10^3

Fig. 5. Effect of hyaluronidase digestion on the elution of component A, chondroitin sulphate, from a column of Sephadex G-25

Component A (1.64 µmol of uronic acid) isolated from tracheal secretions by DEAE-cellulose DE-52 ion-exchange chromatography was applied to a Sephadex G-25 column (38 cm × 1 cm) and 1.7 ml fractions were collected and analysed for uronic acid by the carbazole method. The effect of hyaluronidase digestion carried out as described in the Materials and Methods section on the elution of component C was assessed by direct application of the digestion products to the column and fractions (1.7 ml) were analysed as above. Component A (—); component A after hyaluronidase digestion (---).

papain digestion and ion-exchange chromatography resulted in its complete degradation to tetrasaccharides, as indicated by the elution profile on Sephadex G-25 (Fig. 5). Dermatan sulphate, which is resistant to testicular hyaluronidase, thus appeared to be largely absent from this fraction (Schmidt & Dmochowski, 1964). Analysis of the *N*-acetylhexosamine moieties of the tetrasaccharides recovered from the Sephadex G-25 column (Reissig *et al.*, 1955) suggested that the original fraction consisted of 72% chondroitin 6-sulphate and 28% chondroitin 4-sulphate (Mathews & Inouye, 1961).

The chondroitin sulphate concentration in the medium was about 2.5 times that of hyaluronic acid, despite the ninefold greater specific radioactivity of the hyaluronic acid fraction (Table 3). These differences in concentration and specific radioactivities were not significantly changed during prolonged culture (up to 10 days) with D-[1-¹⁴C]glucosamine present throughout the culture period.

The macromolecule with the highest specific radioactivity isolated from the secretions was the glycoprotein, component C (5.4×10^3 c.p.m./µmol of hexosamine), reflecting the high metabolic activity of the epithelial layer (Table 3).

Table 4. G.l.c. analysis of the insoluble mucus and the soluble glycoprotein (component C)

The insoluble mucus was separated from the medium by centrifugation, freeze-dried and analysed directly. The soluble glycoprotein (component C) was isolated from the medium after digestion with papain and DEAE-cellulose DE-52 fractionation. Results shown are the average value of two separate preparations. Small amounts of mannose and xylose were always present in component C.

Monosaccharide	Molar ratios of sugars present (<i>N</i> -acetylgalactosamine = 1)	
	Insoluble mucus	Soluble glycoprotein (component C)
Fucose	0.47	0.45
Galactose	1.58	1.20
<i>N</i> -Acetylgalactosamine	1.00	1.00
<i>N</i> -Acetylglucosamine	1.09	1.43
Sialic acid	0.81	1.25
Sialic acid: fucose ratio	1.72	2.78

Analysis of insoluble mucus

G.l.c. analysis of the insoluble mucus showed that the monosaccharide content was characteristic of epithelial glycoproteins, and the molar ratios of the sugars were similar to those in the soluble glycoprotein fraction of culture medium (Table 4). Insoluble mucus was readily solubilized by papain digestion, and a trichloroacetic acid-soluble fraction was isolated which had an electrophoretic mobility identical with that of the papain-digested soluble glycoprotein. This fraction, which was eluted in the void volume on Sephadex G-200 gel filtration, was radioactive when cultures were incubated with Na₂³⁵SO₄ and D-[1-¹⁴C]glucosamine.

Discussion

In vertebrates, glycoproteins are found as mucins endowing secretions of the epithelial mucosa with the high viscosities and the capacity for forming viscoelastic gels essential to functions of protection and lubrication (Gibbons, 1972). Although mucus from a wide variety of sources has been studied in detail, tracheal mucus has received little attention, mainly because of the difficulties involved in obtaining samples which are not contaminated with saliva. Holden *et al.* (1971a,b) overcame this problem by developing a tracheal pouch system in dogs, from which they successfully separated glycoproteins by using agarose-gel electrophoresis after reduction with dithioerythritol. Organ-culture techniques also offer the probability of collecting uncontaminated tracheal mucus, as shown by the results of Ellis & Stahl (1973a,b), who isolated glycoproteins from dog tracheal explants similar in character to those produced

by dog tracheal pouches. Those cultures did not appear to give rise to connective-tissue components in the culture medium; however, rabbit tracheal cultures used in the present study were not so accommodating in this regard, giving rise to extracellular macromolecules from both epithelial and connective-tissue cells.

Isolation and characterization of the secretions were carried out after digestion with papain, which decreased losses of soluble glycoproteins during DEAE-cellulose fractionation. Structural alterations in the glycoprotein components resulting from papain treatment have not been investigated in detail, apart from the observed slight increase in electrophoretic mobility. The action of papain lowered the viscosity of the secretions, a factor which presumably facilitated recovery of the glycoproteins from the ion-exchange column. Proteolytic enzymes have been used successfully by a number of other workers when characterizing glycoproteins in mucous secretions (see, e.g., Draper & Kent, 1963; Snary & Allen, 1972), particularly when problems of highly viscous fluids and insoluble gels are involved.

Snary & Allen (1972) have shown that the soluble and insoluble glycoproteins found in pig gastric mucin were identical, whereas agarose-gel electrophoresis of comparable fractions from dog tracheal explants indicated that different glycoproteins were involved (Ellis & Stahl, 1973a). Glycoproteins secreted by rabbit tracheal cultures were also found in both soluble and insoluble fractions which, after papain digestion, had a number of features in common in that they were both high-molecular-weight compounds of similar electrophoretic mobility and both were sulphated, as shown by their incorporation of radioactivity after incubation with $\text{Na}_2^{35}\text{SO}_4$. Their carbohydrate compositions had certain similarities (Table 4), particularly their high sialic acid:fucose ratios, but the overall molar ratios of the sugars present showed variations which cannot yet be reconciled with the conclusion that they are identical molecules. The mannose found in the soluble glycoprotein (Table 4) indicated that a small amount of non-epithelial glycopeptide was present in this fraction (Marshall, 1972), which could distort the measured sugar ratios. Further purification of this component is necessary to resolve these problems.

Trachea being a cartilage-containing tissue, the presence of chondroitin sulphate in the culture fluid was predictable, but the detection of hyaluronic acid in such relatively large amounts was unexpected. Hyaluronic acid has been identified in pig laryngeal cartilage (Hardingham & Muir, 1974), but in much smaller amounts than in rabbit tracheal secretions. It seems probable therefore that the major portion of the secreted hyaluronic acid has a different cellular origin from chondroitin sulphate, a view supported by

the finding that their specific radioactivities differ widely (Table 3). It is presumed that connective-tissue fibroblasts located beneath the epithelium and between the cartilage rings are the principal source of tracheal hyaluronic acid; in this connexion it is noteworthy that no hyaluronic acid is detectable in the secretions from the perfused tracheal lumen of cats, which are derived almost entirely from the epithelial mucosa (J. T. Gallagher, P. W. Kent, P. Richardson & J. Widdicombe, unpublished work).

The complexity of the tracheal organ-culture system is emphasized by the findings of Wiebkin & Muir (1973) that hyaluronic acid in low concentrations ($0.05 \mu\text{g/ml}$) inhibits chondroitin biosynthesis in isolated chondrocytes. We have no information at present on the possibility of the inhibitory effect of the high concentrations in the medium of tracheal hyaluronic acid on the synthesis and secretion of tracheal chondroitin sulphate. In the organ-culture system, the extracellular matrix is intact and should preclude direct access to the chondrocyte membrane, which is supposedly necessary for hyaluronic acid to exert its inhibitory effect.

The broad molecular size distribution (Fig. 3) of chondroitin sulphate in the native secretions suggested that it was present in the form of proteoglycan. However, it could not be detected in the V_0 fraction from Sepharose 2B, and it thus appeared to be of lower average molecular weight than proteoglycans extracted with dissociative solvents from pig larynx (Hardingham & Muir, 1972) and rat costal cartilage (Wasteson *et al.*, 1972). This may indicate that partial degradation of the proteoglycan molecule precedes its release from the cartilage matrix. Wasteson *et al.* (1972) demonstrated that the release of proteoglycan from rat costal cartilage in culture is accompanied by a decrease in molecular weight.

Fractionation on Sepharose 2B also showed that proteoglycan and hyaluronic acid in the culture medium do not interact to produce the aggregates of large hydrodynamic size as are formed when human umbilical-cord hyaluronic acid is mixed with pig laryngeal proteoglycan (Hardingham & Muir, 1974). Their failure to interact may have been due to the postulated degradative change in the proteoglycan molecule, and it is conceivable that modification of proteoglycan structure to decrease or abolish its capacity for associating with hyaluronic acid is an essential step in the turnover of the cartilage matrix.

The high incorporation of radioisotopes into epithelial glycoproteins and connective-tissue glycosaminoglycans, which were in turn secreted into the medium at constant rates over at least 9 days in culture, indicated that rabbit tracheal explants in culture could be valuable for studying many current problems relating to these complex substances, particularly the control of mucous secretion and the metabolism of

the extracellular matrix. One explanation for the reproducibility of these cultures may be that the constant activity of the ciliated epithelium promotes oxygenation of the system and maintains a steady circulation of medium which prevents accumulation of high local concentrations of toxic metabolites.

We thank the Cystic Fibrosis Research Foundation Trust and the Medical Research Council for their generous financial support.

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