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Biosynthesis of Respiratory-Tract Mucins

INCORPORATION OF RADIOACTIVE PRECURSORS INTO GLYCOPROTEINS BY CANINE TRACHEAL EXPLANTS *IN VITRO*

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1. Canine tracheal explants, cultured in medium 199, actively incorporated radioactive precursors into secreted macromolecules *in vitro*. 2. Puromycin, 6-diazo-5-oxo-L-norleucine and ouabain markedly inhibited the incorporation of these precursors. 3. Exogenous glucosamine at concentrations above 20mM caused a greater than 50% inhibition of the incorporation of L-[G-^{3}H]fucose and L-[U-^{14}C]serine. 4. Carbohydrate content of the purified secretions was approximately 50% and consisted principally of galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucose and sialic acids. 5. Chromatography on DEAE-cellulose and Bio-Gel A-150m and equilibrium density-gradient centrifugation in a CsCl gradient confirmed the presence of mucous glycoproteins. 6. Electrophoresis on 1% agarose gels gave profiles that were identical with canine respiratory mucus obtained *in vivo*. 7. These results support the utility of the explant system for studies of respiratory secretions.

The mammalian respiratory tract is protected by mucous secretions that originate from the epithelial goblet cells and the submucosal tracheobronchial mucous glands. These secretions, in addition to maintaining the normal function of the respiratory tract, are important factors in chronic obstructive lung diseases (Yeager, 1971). To understand their biological role and the relationship between secretion and the development of disease it is necessary to isolate and characterize the macromolecular components of these secretions. The principal non-diffusible components of respiratory expectorations have been shown to be mucous glycoproteins (Havez & Biserte, 1968). Despite the importance of these glycoproteins little is known concerning their structure, synthesis and secretion.

The majority of studies of respiratory tract mucins have been histochemical and radioautographic (Havez *et al.*, 1967; Havez & Biserte, 1968; Lamb & Reid, 1969; Sturgess & Reid, 1972). Respiratory expectorations in diseased states have also been extensively studied (Havez *et al.*, 1967; Ryley & Brogan, 1968). Other investigators have examined tracheobronchial tissues for their ability to incorporate radioactivity into trichloroacetic acid-precipitable material after incubation in radioactively labelled culture medium (Formijne *et al.*, 1964; Yeager *et al.*, 1971).

More recently studies have been made of the macromolecules secreted by tracheobronchial explants (Kent *et al.*, 1969; Stahl & Ellis, 1970). The experiments described in the present paper are concerned with the further development of these techniques, together with the fractionation and characterization of the radioactive macromolecules. Preliminary reports have been published (Stahl & Ellis, 1970; Ellis & Stahl, 1971).

Experimental

Materials

Tissue culture medium 199 with Earle modified salts solution was supplied by Grand Island Biological Co., Grand Island, N.Y., U.S.A. Antibiotics and puromycin were purchased from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Glucosamine, ouabain and dithioerythritol were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., $CaCl_2$ (optical grade) and urea (ultra pure) from Schwarz–Mann, Orangeburg, N.Y., U.S.A. 6-Diazo-5-oxo-L-norleucine was prepared by the method of DeWald & Moore (1958). DEAE-cellulose (Whatman DE-32; 1mequiv./g dry wt.) was obtained from H. Reeve Angel, Clifton, N.J., U.S.A. and pre-cycled as described by the manufacturers. Bio-Gel A-150m was purchased from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Agarose for gel electrophoresis was obtained from Marine Colloids, Inc., Rockland, Maine, U.S.A. All radioactive compounds were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The specific radioactivities of the isotopes were as follows: D-[1-^{14}C]glucosamine, 5–10mCi/mmol;

D-[U-^{14}C]glucose, 1–5mCi/mmol; D-[6-^{3}H]glucosamine, 1–2mCi/mmol; $Na_2{}^{35}SO_4$, 10–1000mCi/mmol; L-[G-^{3}H]fucose, 1–5Ci/mmol, DL-[4,5-^{3}H]-leucine, 5Ci/mmol; L-[U-^{14}C]serine, >100mCi/mmol. Radioactivity was measured in a Nuclear–Chicago liquid-scintillation counter by using scintillation fluid prepared as described by Bray (1960). Radioactivity in gels was measured after slicing the gels into 2mm thick discs and dissolving in 0.5ml of water at 100°C directly in the scintillation vials. When gels had been stained with periodic acid–Schiff reagent, 0.1ml of 30% (v/v) H_2O_2 was added to remove colour before dissolving in water. After solubilizing the gel disc, 10ml of Bray's (1960) scintillation fluid was added.

Methods

Preparation and culture of explants. Organ culture of tracheal and bronchial explants was carried out by the technique described by Hoorn (1966). Trachea and mainstem bronchi were excised from adult, male, mongrel dogs overdosed with pentobarbital sodium and the explants prepared as previously described (Stahl & Ellis, 1972). Explants were cultured in serum-free medium 199 at 37°C, with humidity control and an air+CO_2 (95:5) atmosphere regulating the pH between 7.1 and 7.3, for periods of 24–72h. Under these conditions ciliary activity and secretion of macromolecules continued for at least 1 week although some morphological changes in the submucosal glands occur after 96h. Histological monitoring of epithelial cells indicated no gross morphological changes during 96h. When radioactive precursors were used they were present in the incubation medium at the following concentrations; D-[1-^{14}C]glucosamine (0.5μCi/ml of medium), D-[U-^{14}C]glucose (1μCi/ml), D-[6-^{3}H]glucosamine (2μCi/ml), $Na_2{}^{35}SO_4$ (2μCi/ml), L-[G-^{3}H]fucose (1μCi/ml), DL-[4,5-^{3}H]leucine (2μCi/ml) and L-[U-^{14}C]serine (1μCi/ml).

Collection and initial preparation of secretions. At the completion of the incubation period the explants were washed with the incubation medium and then rinsed with fresh 199 culture medium (at 37°C) to free adhering mucus strands. The mucus suspension, consisting of spent culture fluid, mucus strands and washings, was treated in one of several ways. For chromatographic analysis the suspension was made 6M with respect to urea. This solution was then passed through a Sephadex G-200 column (2.5cm×30cm) equilibrated with 6M-urea. For gel-electrophoresis experiments the mucus suspension was centrifuged at 30000*g* for 30min to give a pellet (the insoluble phase of the secretion). The supernatant remaining was then dialysed and freeze-dried.

Analytical procedures. The protein concentration of column effluent fractions was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Carbohydrate-positive material eluted from columns was determined by the phenol–H_2SO_4 method of Dubois *et al.* (1956). Hexuronic acid was estimated by the procedure of Bitter & Muir (1962) with glucuronolactone as a standard. The amino acid composition of the purified secreted material was determined with a Phoenix Instruments amino acid analyser after hydrolysis in 6M-HCl at 110°C for 20h under N_2. Individual neutral and amino sugars were determined by the g.l.c. method of Griggs *et al.* (1971) after 3M-HCl hydrolysis at 100°C for 3h and conversion into the corresponding alditol acetates. Sialic acids were measured by the thiobarbituric acid method of Warren (1959) after hydrolysis with 0.05M-H_2SO_4 for 60min at 100°C, with *N*-acetylneuraminic acid as standard. SO_4^{2-} was determined colorimetrically at 642nm by using Sulphonazo III after hydrolysis in 1M-HCl for 16h at 100°C (Schrager & Oates, 1970).

Gel chromatography. The Sephadex G-200 void volume peak was further chromatographed on a Bio-Gel A-150m column (2.5cm×40cm) equilibrated and eluted with a 0.2M-Tris–potassium phosphate buffer, pH7.0, containing 0.02% sodium azide and 6M-urea at 4°C. Each fraction was assayed for radioactivity and hexose content.

Ion-exchange chromatography. Fractionation on DEAE-cellulose was carried out as described by Gernez-Rieux *et al.* (1963), either before or after gel chromatography on 1% agarose. A column (2.5cm×5.5cm) was packed with a slurry of DEAE-cellulose suspended in 5mM-potassium phosphate buffer, pH8.0. The column was then washed with 100ml of buffer containing 6M-urea. The mucus sample was applied to the column and eluted in a stepwise manner, with 3 column volumes each of 5mM-potassium phosphate buffer, pH8.0; 0.15M-potassium phosphate, pH5.5; 0.3M-potassium phosphate, pH4.3; 0.3M-sodium carbonate and finally 0.1M-NaOH. All buffers were made up in 6M-urea. The phosphate buffers were adjusted to final pH by addition of either 1M-KOH or 1M-phosphoric acid. The hexose and radioactive contents of 5ml fractions were determined.

Gel electrophoresis. Mucus samples, previously separated by centrifugation, were reduced with 0.5% dithioerythritol and subjected to electrophoresis on 1% agarose gels as described by Holden *et al.* (1971*b*). The system essentially involved running the reduced samples in 1% agarose gels containing 0.1% sodium dodecyl sulphate in a 0.09M-Tris–0.0025M-EDTA–0.09M-sodium borate buffer at pH8.2. Final buffer pH was achieved with the addition of 1M-HCl. A typical run was for 35 to 45min at a constant current of 5mA/tube in 75×5mm internal diameter tubes. After electrophoresis the

gels were removed from their tubes and stained for protein and carbohydrate. Protein bands were located by soaking the gels in a 0.25% solution of Coomassie Blue in 7.5% (v/v) acetic acid. Periodic acid–Schiff reagent was used to locate carbohydrate bands as described by Holden *et al.* (1971*a*).

Equilibrium density-gradient centrifugation. Radioactively labelled explant secretion dissolved in a 6M-urea–0.2M-Tris–0.05M-potassium phosphate buffer, adjusted to pH7.0 with 1.0M-phosphoric acid, was mixed with solid CsCl until a density of 1.40–1.45g/ml was obtained. CsCl equilibrium density-gradient centrifugation was performed as described by Creeth & Denborough (1970). The sample was placed in a 5ml polycarbonate tube in the SW 50 rotor and centrifuged at 42000rev./min for 60h at 10°C in a Beckman model L2 ultracentrifuge. At the end of the centrifugation, a hole was pierced in the bottom of the tube and 0.35ml fractions were collected. The density of the fractions was calculated from the results of weighings made with a calibrated 200μl pipette both before and after filling.

Results

Metabolism of canine tracheal explants

Radioautographic studies (Havez *et al.*, 1967; Lamb & Reid, 1969; Yeager *et al.*, 1971) have shown that exogenous amino acids, carbohydrates and SO_4^{2-} are incorporated into the mucus-producing structures of the tracheobronchial tree. The present studies were designed to demonstrate that the labelled macromolecules are secreted by these structures as mucin-type glycoproteins.

Tracheal explants were incubated in the presence of radioactive precursors for periods of up to 72h. The metabolic activity of the tissues was determined by measuring the incorporation of radioactivity in acid-precipitable material released into the medium. Samples of the culture medium were treated with 5% trichloroacetic acid–1% phosphotungstic acid and the secretion rate/24h calculated. The explants were viable throughout the 72h incubation period as determined by secretion rates of labelled macromolecules/24h incubation period. For labelled glucosamine, SO_4^{2-} and amino acids the specific radioactivity of the secreted macromolecules was still slightly increasing after 72h. This is presumably due to the changing specific radioactivities of precursors within the tissues and the finite time for the macromolecules to be synthesized and secreted. The metabolic activity of the explants, as measured by secretion rate, did not appear to be affected by the presence of antibiotics in the culture medium.

As an alternative to acid precipitation, secreted macromolecules were separated from the spent culture fluid by chromatography on Sephadex G-200. Material eluting in the void volume of the column was taken to be macromolecular in nature. This procedure was used to investigate the effects of several compounds on the synthesis and secretion of mucin macromolecules. Puromycin, present in the culture medium at a concentration of 2mM, caused 90% inhibition of the incorporation of labelled amino acids, glucosamine and SO_4^{2-} into the Sephadex G-200 void-volume material over a 24h incubation period. Ouabain (0.1mM) decreased the amount of labelled macromolecules secreted into the medium by 8%. Gallagher & Marsden (1969) reported a similar effect with submaxillary-gland cell cultures. 6-Diazo-5-oxo-L-norleucine (0.1mM), a potent inhibitor of L-glutamine–D-fructose 6-phosphate aminotransferase (Ellis & Sommar, 1972), decreased the incorporation of labelled glucose and SO_4^{2-} by over 85%. High concentrations of glutamine also inhibit the tracheal aminotransferase (Ellis & Sommar, 1971). Increasing the concentration of glutamine in medium 199 from 0.67 to 20mM resulted in a 40–50% decrease in the incorporation of radioactivity from D-[U-^{14}C]-glucose into mucin macromolecules. The effects of increased concentrations of glucosamine were also studied (Table 1). In this experiment the rates of incorporation of radioactive fucose and serine into secreted macromolecules in a given time-period were used as measures of the metabolic activity of the explants in the presence of 20mM- and 50mM-glucosamine.

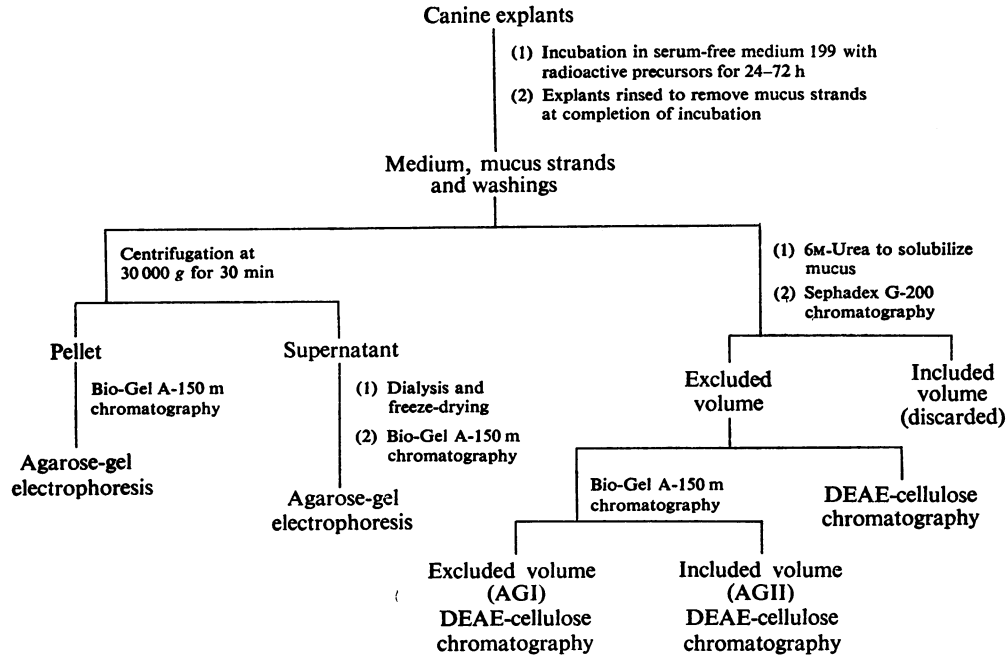
Fractionation and characterization of the secreted macromolecules

As an aid in characterization of the secreted labelled macromolecules as mucous glycoproteins, comparative studies were carried out with mucus obtained

Table 1. *Effect of glucosamine on incorporation of* L-[*G*-^{3}*H*]*fucose and* L-[*U*-^{14}*C*]*serine into substances secreted by canine tracheal explants, which elute at the void volume of Sephadex G-200 columns*

Explants were incubated as described under 'Methods'. Results are average values from two experiments at each glucosamine concentration and are expressed as d.p.m. incorporated into macromolecules/24h incubation period.

Concentration of exogenous glucosamine (mM)	10^{-3} × Radioactivity of secreted macromolecules (d.p.m./g of explant)	
	L-[G-^{3}H]Fucose	L-[U-^{14}C]Serine
0	376	264
20	128	154
50	81	95



Scheme 1. *Flow diagram of experimental procedures for separation and characterization of tracheal explant secretions*

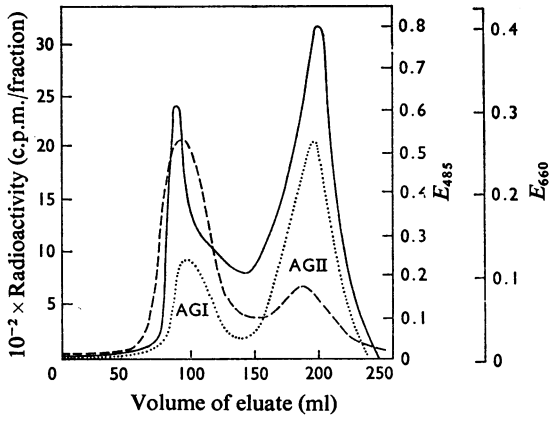


Fig. 1. *Bio-Gel A-150m chromatography of* D-[1-^{14}C]-*glucosamine-labelled explant secretions*

A portion of the Sephadex G-200 void-volume fraction was loaded on a Bio-Gel A-150m column (2.5cm ×40cm), eluted with 0.2M-Tris containing 6M-urea and with pH adjusted to7 with phosphoric acid by downward flow. Fractions (3ml) were collected. The column void volume was 80ml. ——, Radioactivity; – – – –, carbohydrate as determined by the phenol–H_2SO_4 method (E_{485}); ·····, protein as determined by the Lowry *et al.* (1951) method (E_{660}).

in vivo from a canine tracheal pouch (Wardell *et al.*, 1970). The Sephadex G-200 void-volume material was further fractionated by gel filtration on 1% agarose columns and by DEAE-cellulose chromatography. A summary of the procedures used in these studies is shown in Scheme 1. Canine tracheal pouch mucins obtained *in vivo* have a characteristic elution profile when fractionated on Bio-Gel A-150m by continuous elution with 0.2M-Tris–potassium phosphate, pH7, in 6M-urea (Holden *et al.*, 1971*b*). In the hope of detecting a similar secretion of labelled glycoproteins by tracheal explants, the Sephadex G-200 void-volume fraction from explant secretion was concentrated and applied to a Bio-Gel A-150m column. Two distinct fractions were obtained with coincident profiles of radioactivity, phenol–H_2SO_4-positive material and protein as determined by the method of Lowry *et al.* (1951) (Fig. 1). The fraction AGI, which was eluted just after the void volume of the column, contained about 45% of the D-[1-^{14}C]glucosamine label and a majority of the carbohydrate-positive material. Fraction AGII, a more disperse fraction eluting near the total volume of the column, contained most of the Lowry-positive material. The radioactive material present in the AGI fraction corresponded to the large-molecular-weight glycoproteins of canine tracheal mucins described by Holden *et al.* (1971*b*). Secre-

tions obtained *in vitro* and labelled with different radioactive precursors were similarly subjected to gel filtration. With all fractionations on Bio-Gel A-150m over 90% of the radioactivity originally applied to the columns was recovered.

Distribution of radioactivity between the two fractions, AGI and AGII, varied depending upon the labelled precursor. The amount of radioactivity found in fraction AGI, expressed as a percentage of the total label eluted from the column, was 46% for D-[1-^{14}C]glucosamine, 65% for $Na_2{}^{35}SO_4$, 18% for L-[G-^{3}H]fucose and 17% for DL-[4,5-^{3}H]leucine. These results indicate that fraction AGI is a glycoprotein, which is relatively rich in SO_4^{2-} and appears to be of large molecular weight or aggregates strongly.

The two Bio-Gel fractions were concentrated, dialysed and then further chromatographed on DEAE-cellulose as described for respiratory expectorations by Gernez-Rieux *et al.* (1963). Elution from these columns was with 3 bed volumes of each buffer as described under 'Methods'. The distribution of radioactivity from the Bio-Gel peaks between the DEAE-cellulose fractions is given in Table 2. The material eluted by 0.3M-sodium carbonate is the major component of both fractions AGI and AGII.

Table 2. *Fractionation of* D-[1-^{14}C]*glucosamine-labelled Bio-Gel A-150m peaks, AGI and AGII, by DEAE-cellulose chromatography*

The two Bio-Gel peaks were pooled, concentrated, and a portion of each placed on a DEAE-cellulose column equilibrated with 0.005M-potassium phosphate buffer, pH8.0, containing 6M-urea. Elution was stepwise with 3 column volumes of each buffer. Results are expressed as the percentages of radioactivity found in each DEAE-cellulose fraction.

DEAE-cellulose eluant buffer	% of Bio-Gel radioactivity eluted by each DEAE-cellulose buffer	
	Fraction AGI	Fraction AGII
0.005M-Potassium phosphate, pH8	—	8
0.15M-Potassium phosphate, pH5.5	—	8
0.3M-Potassium phosphate, pH4.3	—	11
0.3M-Sodium carbonate	72	70
0.1M-NaOH	28	3

Table 3. *Chemical composition of purified AGI fraction from canine tracheal explant secretions*

Experimental details are described in the text. Results are expressed as mol/100mol of amino acids or monosaccharides released by acid hydrolysis of tracheal secretions dried to constant weight. Amino sugars were presumed to occur as the *N*-acetyl derivatives.

Component	Amount (mol/100mol of amino acid)	Component	Amount (mol/100mol of monosaccharides)
Aspartic acid	10.1	Fucose	18.2
*Threonine	7.3	Galactose	34.8
*Serine	7.6	*N*-Acetylglucosamine	21.7
Glutamic acid	9.5	*N*-Acetylgalactosamine	16.0
Proline	9.5	*N*-Acetylneuraminic acid	6.0
Glycine	8.8	Mannose	1.7
Alanine	7.3	Glucose	0.6
Cystine (half)	2.5	Uronic acid	0.8
Valine	6.9		
Methionine	1.6	Sulphate	13.0
Isoleucine	3.8		
Leucine	7.9		
*Tyrosine	2.8		
Phenylalanine	3.5		
Lysine	4.7		
Histidine	1.9		
Arginine	4.4		

* No correction was made for destruction during acid hydrolysis.

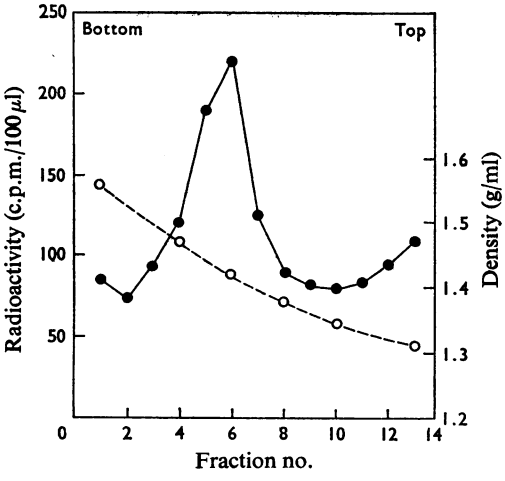


Fig. 2. *CsCl equilibrium density-gradient centrifugation of* D-[1-^{14}C]*glucosamine-labelled explant secretions*

[^{14}C]Glucosamine-labelled secretions, prepared as described in the Experimental section were centrifuged for 60h at 42000rev./min at 10°C. The gradient was fractionated by puncturing the bottom of the tube and collecting 0.35ml fractions dropwise. ●, D-[1-^{14}C]Glucosamine radioactivity; ○, density.

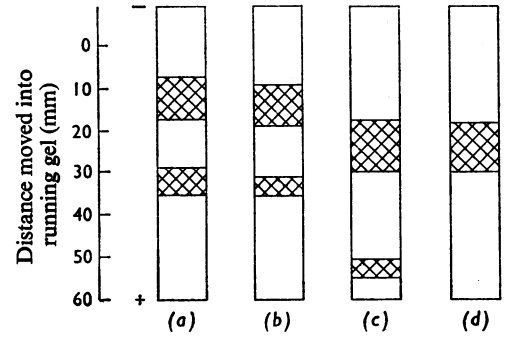


Fig. 3. *Diagram of agarose-gel-electrophoresis patterns of canine respiratory mucins*

The mucin fractions were solubilized with dithioerythritol and subjected to electrophoresis in 1% agarose gels containing sodium dodecyl sulphate at pH8.2. Gels were run at a constant current of 5mA/gel for 45min. Approx. 6V/gel was required to develop this current. The sample gel is at the top of each gel (cathode) and glycoprotein bands were located by staining for carbohydrate with periodic acid–Schiff reagent. (*a*), Explant secretion pellet; (*b*), tracheal pouch mucus; (*c*), explant secretion supernatant; (*d*), intact airway mucus.

This fraction has been characterized as mucin-type glycoproteins by Gernez-Rieux *et al.* (1963) by using gel electrophoresis, paper electrophoresis and immunoelectrophoresis, whereas the material eluted by the first three buffers was identified as plasma-type glycoproteins and mucopolysaccharides. The percentage of the radioactivity incorporated into secreted macromolecules eluting with 0.3 M-sodium carbonate after chromatography on DEAE-cellulose was 72% for D-[1-^{14}C]glucosamine, 80% for $Na_2{}^{35}SO_4$, 65% for L-[G-^{3}H]fucose and 30% for DL-[4,5-^{3}H]leucine.

Amino acid and carbohydrate analyses of the purified AGI fraction macromolecules are given in Table 3. The protein content of the material, both by amino acid composition and by the colorimetric method of Lowry *et al.* (1951), was about 48%. Seventeen amino acids were identified. Aspartic acid, threonine, serine, glutamic acid, proline, glycine and alanine were present in the largest amounts as has been reported for other tracheobronchial secretions (Havez *et al.*, 1968; Roussel *et al.*, 1972). The carbohydrate content of the purified fraction was approximately 40% expressed on a dry-weight basis. *N*-Acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose and sialic acid were the major sugars in the molar proportions 1.0:1.36:2.2:1.14:0.38 with a minor amount of mannose, glucose and uronic acid indicating some small contamination by other macromolecules. The mucin also contained $SO_4{}^{2-}$ (13mol/100mol of monosaccharides).

The buoyant density of proteins in aqueous CsCl is approx. 1.3g/ml, that of carbohydrates is approx. 1.6g/ml and glycoproteins exhibit a buoyant density intermediate between these two extremes depending upon the carbohydrate content (Creeth & Denborough, 1970). Freeze-dried explant secretions labelled with D[1-^{14}C]glucosamine were solubilized in 6M-urea and mixed with solid CsCl to give an initial density of 1.40–1.45g/ml. Equilibrium density-gradient centrifugation was carried out as described by Creeth & Denborough (1970). The final CsCl density was 1.32g/ml at the top of the tube and 1.56g/ml at the base of the tube. The D-[1-^{14}C]-glucosamine radioactivity formed a sharp peak with a density of 1.42g/ml giving complete separation from extraneous protein (Fig. 2).

Holden *et al.* (1971*a*,*b*) used polyacrylamide–agarose and pure agarose gels to separate various mucins by electrophoresis. Further characterization of the explant secretions as mucin-type glycoproteins was carried out by electrophoresis on 1% agarose gels. Explant secretions were compared with canine pouch mucus, and with canine respiratory tract mucus obtained by bronchoscopy after ether induction. In this series of gel-electrophoresis experiments the explant secretions were initially separated

by centrifugation, before being reduced with dithioerythritol and then subjected to electrophoresis. The mucin pellet *in vitro* on electrophoresis gave a profile identical with that obtained with canine tracheal pouch mucus (Fig. 3). The supernatant fraction of the secretion exhibited an electrophoretic profile unlike the pouch mucus but resembled in part the pattern of mucus obtained from the intact canine airway by bronchoscopy (Fig. 3). Radioactivity from D-[1-^{14}C]glucosamine and $^{35}SO_4^{2-}$ was incorporated into the faster-moving band of the pellet fraction and into both bands of the supernatant fraction.

When secretions obtained from brushed explants (Stahl & Ellis, 1972) are subjected to electrophoresis, radioactivity is incorporated into the slower-moving band of the pellet fraction. Further studies illustrating differences between epithelial goblet cell secretions and those originating from the submucosal glands are described in the following paper (Stahl & Ellis, 1973). The fast-moving band of the supernatant fraction was identified as an AGII component by chromatography on Bio-Gel A-150m. This band does not appear in the bronchoscopy sample which consisted only of AGI material.

Discussion

The secretions of the mammalian tracheobronchial tree are important to respiratory function. The physicochemical properties of these secretions are characteristic of solutions of mucous glycoproteins. From studies of respiratory expectorations these macromolecules have been shown to have structural patterns similar to other epithelial mucins (Havez *et al.*, 1968). Ideally, any study of tracheobronchial mucins ought to be made on material collected directly from the lower respiratory tract rather than on mixed samples such as sputum. However, material collected in this manner is rather scant and we have therefore used the technique of organ culture.

Preparations from a number of mucus-secreting tissues have been shown to be metabolically active *in vitro* and to incorporate radioactivity from suitable radioactive precursors into glycoproteins. Such studies on tracheobronchial mucins were primarily confined to extracts of epithelial tissues (Formijne *et al.*, 1964; Lamb & Reid, 1969; Yeager *et al.*, 1971). This demonstration of a macromolecule containing carbohydrate in tissue extracts does not prove that the material is actually a secretory product. More recently the incorporation of labelled precursors into macromolecules secreted into the culture medium has been demonstrated (Ellis & Stahl, 1971; Kent *et al.*, 1971).

The present investigations confirm the usefulness of studies made with tracheal organ cultures *in vitro* and also demonstrate the presence of mucous glycoproteins in the secretions from such tissues. The incorporation of labelled precursors, including sugars amino acids and SO_4^{2-}, into secreted macromolecules continued throughout a 72h incubation period. These results indicate that both synthesis and secretion can be studied in isolated explants over an extended incubation period. The incorporation of labelled serine and fucose into macromolecules discharged by the mucus-synthesizing structures of the tracheobronchial tree is described here for the first time.

The inhibitory effects of puromycin on the incorporation of labelled precursors suggest that continuing protein synthesis is a prerequisite for the secretion of mucous macromolecules. The 6-diazo-5-oxo-norleucine inhibition of $^{35}SO_4^{2-}$ incorporation into secreted macromolecules could be prevented by exogenously supplied D-glucosamine. This result suggests that incorporation of SO_4^{2-} requires the prior or simultaneous addition of amino sugars to the oligosaccharide side chains of the mucin macromolecules.

The marked inhibition of the incorporation of L-[G-^{3}H]fucose and L-[U-^{14}C]serine by relatively high concentrations of D-glucosamine in the incubation medium demonstrated that the tracheal explants are sensitive to D-glucosamine inhibition of mucin synthesis. This result is of interest since D-glucosamine has been found to inhibit the growth of neoplastic tissues (Quastel & Cantero, 1953; Bekesi *et al.*, 1969*b*) and macromolecular synthesis in tumour cells (Bekesi *et al.*, 1969*a*). This effect of D-glucosamine has been attributed to an accumulation of intracellular UDP-*N*-acetylhexosamine leading to feedback inhibition of the endogenous synthesis of D-glucosamine and a possible impairment in the metabolism of other nucleotides (Bekesi *et al.*, 1969*a*). Tracheal L-glutamine–D-fructose 6-phosphate aminotransferase is subject to feedback inhibition by UDP-*N*-acetylglucosamine (Ellis & Sommar, 1972). The results presented here on the inhibitory effect of endogenous D-glucosamine on explant secretions support the concept that control of the synthesis of glucosamine 6-phosphate and UDP-*N*-acetylglucosamine play an important role in the secretion of mucins by respiratory tissues. The similar inhibitory effect produced by high concentrations of glutamine in the culture medium reflects the glutamine substrate inhibition seen *in vitro* with the tracheal aminotransferase (Ellis & Sommar, 1971).

Fractionation and characterization of the secreted material has demonstrated the presence of mucin-type glycoproteins. The analytical methods employed in the present study have established good correlation between the material obtained *in vitro* and authentic glycoproteins of respiratory mucus obtained *in vivo* (Havez & Biserte, 1968; Holden *et al.*, 1971*a*,*b*; Roussel *et al.*, 1972).

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