Isolation and characterization of lung connective-tissue glycoproteins

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1. Glycoproteins of hamster, rat and baboon lung parenchyma were investigated by using [^{14}C]glucosamine incorporation *in vitro* followed by sequential extraction of the macromolecular components and characterization of the glycoproteins in the extracts. 2. Slices of lung parenchyma maintained *in vitro* incorporated [U- ^{14}C]glucosamine linearly with time into non-diffusible macromolecules for up to 5 h. All the macromolecule-associated ^{14}C label was present as [^{14}C]glucosamine. 3. These ^{14}C -labelled macromolecules were extracted from previously delipidated and salt-extracted lung by 5 M-guanidinium chloride in the presence of dithiothreitol and proteinase inhibitors before (extract A₁) and after (extract A₂) hydrolysis of the collagen by collagenase. The [^{14}C]glucosamine-labelled glycoproteins in extracts A₁ and A₂ contained 55 and 5% respectively of the total [^{14}C]glucosamine incorporated in the lung of all three species studied. 4. The [^{14}C]glucosamine-labelled glycoproteins were analysed by gel-filtration chromatography, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and isoelectric focusing. The major [^{14}C]glucosamine-labelled glycoproteins of baboon lung parenchyma had apparent mol.wts. of about 400 000, 140 000 and 65 000 with isoelectric points respectively of 4.8, 5.4 and 5.4. The hamster lung glycoproteins with isoelectric points of 4.1 and 5.8 were devoid of hydroxyproline and contained galactose, mannose and *N*-acetylglucosamine. These experiments indicate that several distinct glycoproteins are synthesized *in situ* by the cells of pulmonary parenchyma and may well play a role in its structure and function.

The importance of connective tissue in lung structure and function, and the role of alterations in the connective tissue in the production of lung diseases, have been reviewed (Hance & Crystal, 1976; Horwitz *et al.*, 1976; Robert, 1980).

Previous studies on lung connective tissue were mainly related to collagen, elastin and proteoglycans. Little information is available on the glycoproteins of the pulmonary matrix. Such glycoproteins, designated as 'structural glycoproteins' (Robert *et al.*, 1970), were isolated from a variety of connective tissues (Anderson, 1976, 1980; Robert *et al.*, 1976), from lung (Francis & Thomas, 1975; Richmond, 1974, 1981; Darnule *et al.*, 1977) and also from connective-tissue cells in culture (Sear *et al.*, 1977, 1978, 1981; Muir *et al.*, 1976).

Lung glycoproteins designated as 'alveolyns' (Lynn *et al.*, 1979) and containing hydroxyproline

were also isolated from broncho-alveolar lavage and from lamellar bodies of pneumocytes II (Bhattacharya *et al.*, 1975, 1976; Sahu & Lynn, 1979). Ultrastructural and enzymic studies by Vaccaro & Brody (1979) showed the existence of Ruthenium Red-stained filamentous components enmeshed with collagen, possibly glycoprotein in nature.

To obtain further information on glycoproteins of lung connective tissue we investigated their biosynthesis with [^{14}C]glucosamine as a tracer, followed by their extraction with non-destructive methods before enzymic hydrolysis of collagen and their partial characterization. These experiments were carried out on lungs from rats, hamsters and baboons.

Experimental

Materials

Chemicals. [U- ^{14}C]Glucosamine (300 mCi/nmol) was purchased from CEA, Saclay, France. Eagle's

Abbreviation used: SDS, sodium dodecyl sulphate.

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minimum essential medium was obtained from Eurobio, Paris, France; *Vibrio cholerae* neuraminidase was from Behringwerke, Marburg/Lahn, Germany; bovine serum albumin and bovine γ -globulin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (500 i.u./mg) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Acrylamide and *NN'*-methylenebisacrylamide was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Instagel was from Packard, Rungis, France; *N*-ethylmaleimide, phenylmethanesulphonyl fluoride and dithiothreitol were from Merck, Darmstadt, Germany. Ferritin, thyroglobulin, catalase and lactate dehydrogenase were obtained from Pharmacia, Uppsala, Sweden; sucrose solvent was from Beckman, Irvine, CA, U.S.A.; Servalyte was from Serva, Heidelberg, Germany; Dowex X8 was from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Lungs. Lungs were removed from twelve 2-month-old male Sprague-Dawley rats, from nine 1-month-old male golden Syrian hamsters and from a 2-year-old male baboon. Rat and hamster lungs were perfused *in situ* through the pulmonary artery with 0.15 M-NaCl and 0.3% sodium citrate buffer to remove blood, followed by lavage with 0.3% NaCl through the trachea to remove alveolar lipids, proteins and macrophages, and by scraping free of all adhering tissues and from the major airways by dissection. Four baboon lung parenchymal samples (1 ± 0.1 g fresh weight) were withdrawn from the left and right lower lobes and from the left and right upper lobes.

Methods

[U- 14 C]Glucosamine incorporation. The lung samples (1 g) were cut into small fragments of about 1 mm² and incubated at 37°C, with continuous shaking, in Eagle's minimum essential medium (10 ml) containing 10% homologous serum and [14 C]glucosamine (10 μ Ci); streptomycin, penicillin (1 i.u./ml) and mycostatin (100 i.u./ml) were added. The medium was discarded and the tissues washed at 4°C three times with 0.9% NaCl that contained *N*-ethylmaleimide (10 mM), phenylmethanesulphonyl fluoride (2 mM) and EDTA (25 mM) as proteinase inhibitors.

Extractions. Each extraction was carried out in the presence of the proteinase inhibitors. The incubated tissues were homogenized in 1 M-NaCl for 10 s with a tissue homogenizer type 1020 X (International Laboratory Apparatus G.m.b.H., Göttingen, Germany) and shaken with 1 M-NaCl (1:50, w/v) at 4°C as described by Francis & Thomas (1975). The residue was extracted with 2 M-MgCl₂ (1:10, w/v) at 4°C, washed with distilled water until salt-free, delipidated with acetone and

chloroform/methanol (2:1, w/v) for 6 h at 4°C, with continuous shaking, filtered on a sintered-glass funnel and dried *in vacuo*.

The glycoproteins of lung connective tissue were extracted from the defatted dried residue with 5 M-guanidine hydrochloride/0.05 M-dithiothreitol/0.1% EDTA/0.1 M-Tris buffer, pH 8.5, (residue/buffer, 1:30, w/v) by shaking at 20°C for 24 h followed by centrifugation at 35 000 g for 20 min at 15°C (Ross & Bornstein, 1969). The supernatants were designated A₁ extracts. Portions were dialysed: (1) against running tap water and distilled water at 4°C and analysed for hydroxyproline, hexoses, hexosamines and uronic acid; (2) against 6 M-urea/1 M-Tris/0.5 M-EDTA/4% SDS buffer, pH 8, for electrophoresis and isoelectric focusing on polyacrylamide gels; (3) against 6 M-urea, then reduced and carboxamidomethylated by the method of Butler *et al.* (1969) before exclusion chromatography and analytical sucrose-gradient isoelectric focusing.

The residue obtained after extraction with 5 M-guanidine hydrochloride buffer was washed with water until chloride-free and incubated twice with bacterial collagenase (2% of dry weight of the residue) in 1 mM-CaCl₂, 0.1 M-Tris buffer, pH 7.4, at 37°C for 24 h, in the presence of proteinase inhibitors [phenylmethanesulphonyl fluoride (2 mM) and *N*-ethylmaleimide (10 mM)] in the presence of toluene. The absence of non-specific proteolytic activity in the bacterial collagenase preparation was previously checked by incubating ovalbumin with collagenase under the same experimental conditions, but without proteinase inhibitors; no proteolytic activity could be detected. After hydrolysis by collagenase, the lung residues were washed with water until salt-free, and then suspended in 5 M-guanidine hydrochloride buffer, pH 7.4, as described above. The supernatant was designated 'A2'. The final lung residue contained elastin.

Incorporation of [14 C]glucosamine into macromolecules. Portions of extract A₁ were hydrolysed *in vacuo* in 4 M-HCl at 100°C for 4 h and analysed by high-voltage electrophoresis (Moczar *et al.*, 1972) and by t.l.c. (Moczar & Moczar, 1970). To investigate the recovery of 14 C associated with neutral hexoses, portions of proteins were hydrolysed *in vacuo* in 1.5 M-HCl at 100°C for 2 h, neutralized with Amberlite IR 45/OH 40–60-mesh resin and freed of peptide material by passage through a column (0.5 cm $\times$ 4 cm) of Amberlite IR 45/OH resin in the upper part and Amberlite CG resin (H⁺ form; 200–400 mesh) in the lower part; the eluted hexoses were identified by paper chromatography in the solvent system butanol/pyridine/0.1 M-HCl (5:3:2, by vol.) and their radioactivities determined by liquid-scintillation counting. 14 C present as *N*-acetylneuraminate was determined by incubating

portions of extracts with neuraminidase (Klemer & Krasba, 1972) and determining radioactivity in the released [^{14}C] *N*-acetylneuraminic acid.

SDS/polyacrylamide-gel electrophoresis. Electrophoresis was carried out under reducing conditions in the presence of 0.4% SDS and 6M-urea. Polyacrylamide gels were prepared in glass tubes (0.6 int. diam. $\times$ 12 cm). Electrophoresis was carried out at 10°C and at 6 mA/gel. Gels were stained with Coomassie Blue and the periodic acid/Schiff reagent (Fairbanks *et al.*, 1971). Molecular weights were determined as described by Weber & Osborn (1969). Protein standards used were acid-soluble collagen, thyroglobulin, ferritin, bovine γ -globulin, catalase and lactate dehydrogenase. Radioactive glycoproteins were localized by slicing the frozen gel with a parallel array of razor blades, providing samples, 1 or 2 mm thick, followed by digestion of each slice in a scintillation vial with 1 ml of 36% H_2O_2 for 17 h at 37°C. Scintillation fluid (Instagel) was then added and the radioactivity measured after 4 h at room temperature.

Isoelectric focusing. Polyacrylamide-gel isoelectric focusing was carried out under reducing conditions as described by Finlayson (1971) and stained with Coomassie Blue. Reference samples used were cytochrome *c* (mol.wt. 12 800, pI 9.4), haemoglobin (68 000, 7.4 and 7.2), bovine serum albumin (68 000, 4.9), ovalbumin (45 000, 4.6) and Bromophenol Blue (670, 3.0). Glycoproteins were identified by periodic acid/Schiff staining and by the detection of [^{14}C] glucosamine.

Sucrose-gradient isoelectric focusing was performed at room temperature on an LKB 8102 column (LKB Producter, Bromma, Sweden) (1 cm $\times$ 26 cm) with Servalyt (pH range 2–11 or 4–6) with an acidic buffer (0.5% H_3PO_4 in 6M-urea) and basic buffer (0.5% NaOH/6M-urea) and 48% sucrose. Fractions (0.5 ml) were collected for pH measurement and samples were also taken for the determination of radioactivity in 10 ml of sucrose solvent.

Exclusion chromatography. Exclusion chromatography was performed at room temperature on columns (5 cm $\times$ 75 cm) of Sepharose 4B. Descending elution was carried out at a flow rate of 40 ml/h with 6M-urea/0.02M- Na_4EDTA /0.2% SDS, pH 7.5. Fractions (15 ml) were collected and samples were taken for measurement of radioactivity. Dextran Blue 2000 was used to determine the void volume (V_0) and Sudan Red for the total volume (V_t) of the column. Bovine serum albumin (mol.wt. 68 000) and myoglobin (mol.wt. 17 000) were used as standards for molecular-weight determination. ^{14}C -labelled glycoproteins obtained by exclusion chromatography were concentrated on Diaflo PM10 membranes, dialysed against 6M-urea containing Dowex resin (X8; acetate form) to remove SDS and further separated by sucrose-gradient isoelectric focusing.

Analytical methods. Hydroxyproline, hexuronic acids, hexosamines and proteins were determined respectively by the methods of Bergman & Loxley (1963), Blumenkrantz & Asboe Hansen (1973), Blix (1948) and Lowry *et al.* (1951). Amino acid analysis was performed on an LKB autoanalyser, after hydrolysis *in vacuo* of the samples in sealed tubes with 6M-HCl for 24 h. Carbohydrate analyses were performed by g.l.c. (type 104 chromatograph; Pye, Cambridge, U.K.) as described by Zanetta *et al.* (1972).

Results

Extraction

The yield of lung proteins obtained by the outlined extraction procedure is shown in Table 1. The results indicate that the proteins in A_1 and A_2 extracts, in the collagenase digest and in elastin residue represent approx. 25% of the baboon lung dry weight, as was reported also for human lung (Hance & Crystal, 1975). These proteins account only for 15% in the rat and hamster lung, in agreement with previous reports (Collins & Jones, 1978).

Table 1. Extraction of macromolecules of baboon lung and rat lung connective tissue

Extract	Content (mg of protein/100 mg dry weight)			
	Non-collagen proteins*		Collagen†	
	Baboon	Rat	Baboon	Rat
NaCl (1 M)	nd‡	nd	0.20 $\pm$ 0.03	0.14 $\pm$ 0.01
MgCl ₂ (2 M)	nd	nd	0.1 $\pm$ 0.01	0.08 $\pm$ 0.01
Guanidinium chloride (5 M) (extract A_1)	6 $\pm$ 0.5	6 $\pm$ 0.6	2 $\pm$ 0.2	1.1 $\pm$ 0.1
Collagenase digest	nd	nd	19 $\pm$ 0.7	11.2 $\pm$ 0.5
Guanidinium chloride (5 M) (extract A_2)	0.8 $\pm$ 0.1	0.7 $\pm$ 0.1	0	0
Residue (elastin)	3 $\pm$ 1	3 $\pm$ 1		

* Calculated from protein determination (corrected for collagen content) by the method of Lowry *et al.* (1951).

† Calculated from hydroxyproline determination.

‡ Abbreviation used: nd, not determined.

The hexosamine and hexose contents of proteins in A_1 extract were respectively 3.5 and 5 mg/100 mg of protein (average values from the three species investigated). The high ratio of hexosamine to uronic acid (4.3) indicates that glycoproteins are present in higher amount than glycosaminoglycans. The presence of some hydroxyproline suggests the presence of collagen or of collagen-like sequences (Knight *et al.*, 1980) in A_1 extract.

The proteins extracted after hydrolysis of collagen (A_2 extract) contained 5% hexosamine and no hydroxyproline.

Incorporation of [14 C]glucosamine into lung macromolecules

When lung tissue was maintained in Eagle's minimum essential medium, the incorporation of [14 C]glucosamine into glycoproteins extracted by 5 M-guanidinium chloride was linear over a 5 h period (Fig. 1).

Some 85% of the total 14 C radioactivity present in this A_1 extract co-migrated with hexosamines on high-voltage electrophoresis. T.l.c. of hydrolysates showed that 90% of the recovered radioactivity

Fig. 1. Incorporation of [14 C]glucosamine into macro-molecules synthesized by lung fragments incubated *in vitro*

Rat lung cultures containing approx. 100 small fragments per flask, were incubated for 1, 2, 5 or 17 h with [14 C]glucosamine (10 μ Ci) in maintenance medium. For each incubation time, pulmonary fragments in culture were harvested and lavaged, firstly with 0.15 M-NaCl containing 1% glucosamine, and secondly with 0.15 M-NaCl with proteinase inhibitors. The pulmonary fragments were homogenized, extracted with 1 M-NaCl, then with 2 M-MgCl₂ in the presence of proteinase inhibitors, defatted and extracted by 5 M-guanidinium chloride buffer. The radioactivity in this last extract (A_1) was determined. Each point represents the mean of two determinations.

co-migrated with authentic glucosamine. Less than 2% of the total radioactivity chromatographed as hexoses. Less than 10% of the total incorporated radioactivity could be released with neuraminidase.

The distribution of radioactivity in the different extracts was similar in the three species investigated

Fig. 2. Distribution of [14 C]glucosamine (a) in sequential extracts of baboon lung and (b) in successive 5 M-guanidinium chloride extracts (A_2) -

(a) 1, 1 M-NaCl; 2, 2 M-MgCl₂; 3, acetic acid/chloroform; 4, 5 M-guanidinium chloride (A_1); 5, supernatants after hydrolysis of pulmonary residue by collagenase; 6, 5 M-guanidinium chloride (A_2); 7, elastin residue. Each bar represents the mean of five determinations of radioactivity. (b) After hydrolysis by collagenase, the parenchyma residue was subjected to six (1-6) exhaustive extractions with 5 M-guanidinium chloride buffer. Each bar represents the mean of five determinations of radioactivity. 7, Final lung elastin residue.

(Figs. 2a and 2b). About 55% of the incorporated [^{14}C]glucosamine was recovered in the A_1 extracts and only about 5% in the A_2 extracts. About 1% remained in the final residue even after seven successive extractions with 5M-guanidinium chloride buffer, probably because of its strong association with elastin. About 40% of the incorporated [^{14}C]glucosamine was recovered in 1M-NaCl and about 3% could be recovered after hydrolysis of the lung residue with bacterial collagenase. When the collagenase hydrolysis was carried out without inhibitors, 85–88% of the hexosamines, containing 34–47% of the total radioactivity in the supernatants, became dialysable. In the presence of inhibitors, no trace of radioactivity could be detected in the diffusate.

Characterization of glycoproteins of A_1 extract

Polyacrylamide-gel electrophoresis. Fig. 3 shows the incorporation pattern of [^{14}C]glucosamine compared with the distribution of proteins in the gel. Fig. 3 shows the separation pattern of [^{14}C]glucosamine on polyacrylamide-gel electrophoresis. The major Coomassie Blue-staining components could also be stained with periodic acid/Schiff reagent and contained [^{14}C]glucosamine. The corresponding apparent mol.wts. were 140000, 110000 and 35000. A [^{14}C]glucosamine-labelled Coomassie Blue- and periodic acid/Schiff-stained component did not enter into the 5% gel. In 3% gel this component had an apparent mol.wt. of 400000.

Isoelectric focusing on polyacrylamide gel. The radioactivity-incorporation patterns and 'proteinograms' are shown in Fig. 4(a). They were analogous for all three species. The major components of the A_1 extract stained with Coomassie Blue were labelled with [^{14}C]glucosamine. They focused at pI 8.4, 5.5 and 4.5. A higher radioactivity was observed at the level of the pI 8.4 band. When the A_2 extracts were investigated by isoelectric focusing (Fig. 4b), only the components with an acidic pI were labelled with [^{14}C]glucosamine.

Exclusion chromatography. The elution profile (on Sepharose 4B) of the proteins (A_1 extract) from baboon lung is shown in Fig. 5(a). The excluded peak accounts for about 2% of total radioactivity. The fractions (I, II, III) represented 98% of the applied radioactivity. The respective molecular weights calculated from the elution volume were 400000, 140000 and 35000.

The major [^{14}C]glucosamine-labelled and periodic acid/Schiff-positive component of fraction I did not enter into the 4.2% gel on polyacrylamide-gel electrophoresis. Fraction II separated into two major [^{14}C]glucosamine-labelled glycoproteins staining with periodic acid/Schiff reagent, with apparent mol.wts. of 140000 and 65000. The major [^{14}C]glucosamine-labelled glycoprotein of fraction III had

Fig. 3. SDS/polyacrylamide-gel electrophoresis of macromolecules extracted with 5M-guanidinium chloride (A_1 extract)

Macromolecules from extract A_1 were dialysed against 4% SDS/0.5M-EDTA/6M-urea/1M-Tris/HCl buffer, pH 8.0, and processed as described in the text for electrophoresis on gels containing 4.2% (w/v) acrylamide. A portion of the reduced and denatured macromolecules was electrophoresed and stained with Coomassie Blue (—) or periodic acid/Schiff reagent (---); another gel was sliced and assayed for radioactivity (●). The mobilities of the following standards are indicated: 1 and 6, ferritin; 2, collagenase; 3, albumin; 4, catalase; 5, lactate dehydrogenase. The mobilities of the radiolabelled glycoproteins are indicated by arrows.

an apparent mol.wt. of 35000. Each fraction contained hydroxyproline in about equal amounts of 2mg/100mg.

Sucrose-gradient isoelectric focusing. From fraction H of hamster lung (Fig. 5b), two [^{14}C]glucosamine-labelled components were focused at pI 4.1 and 5.8 (Fig. 6a). The component with pI 4.1 did not enter the 4.2% gel on polyacrylamide-gel electrophoresis and its molecular weight was estimated to be 400000 on a 3% gel. The apparent molecular weight of the component with pI 5.6 was 65000 in a 4.2% gel. No precipitation of glycoproteins occurred at the isoelectric point. A non-radioactive peak focused at pH 8.4.

Fig. 4. Isoelectric focusing on polyacrylamide gel of macromolecules extracted with 5 M-guanidinium chloride. Macromolecules from extracts A₁ (a) or A₂ (b) were dialysed against 8 M-urea and processed as described in the text for isoelectric focusing on gels containing 3.7% (w/v) acrylamide. The same portion of the reduced macromolecules was focused, stained with Coomassie Blue (—) and assayed for radioactivity (●). The focusing positions of standards are indicated as follows: 1, cytochrome c; 2, haemoglobin; 3, ovalbumin; 4, Bromophenol Blue. The focusing positions of radiolabelled glycoproteins are indicated by arrows.

From fraction I of baboon lung (Fig. 5a) a radiolabelled glycoprotein, GP I, was focused at pI 4.8 (Fig. 6b). The major radiolabelled glycoproteins, GP II and GP III, from fractions II and III respectively, were focused at pI 5.4 (Fig. 6c). Refocusing of this material over the pH range 4–6 did not change the pI of these major glycoprotein components. The focused glycoproteins were recovered as a precipitate at their isoelectric points. GP II migrated with apparent mol.wts. of 140 000 and 65 000 on polyacrylamide-gel electrophoresis in the presence of SDS. The presence of two bands in the baboon GP II fraction is probably due to an incomplete separation of these two glycoproteins (140 000 and 65 000 mol.wt.) during sucrose-gradient isoelectric focusing. GP III migrated with an apparent mol.wt. of 35 000. From fractions I, II III of baboon lung, a non-radioactive peak was focused at pI 8.4. On the basis of results from sucrose-gradient isoelectric focusing and polyacrylamide-gel electrophoresis, we can assume that at least three glycoprotein components of baboon lung could be separated from the A₁ extract.

Amino acid and carbohydrate compositions. The amino acid composition of glycoproteins of hamster lung (cf. Fig. 6a) with pI of 4.1 and 5.8 are given in

Table 2. Hydroxyproline was not detectable. They were rich in polar amino acids (aspartic and glutamic acid) and contained low amounts of methionine and cysteine. The carbohydrate composition expressed as a molar proportion of galactose, mannose and acetylglucosamine were respectively 2:2:2 for the glycoprotein with pI 4.1 and 2:1:2 for the glycoprotein with pI 5.8.

Table 2 shows also the amino acid compositions of the radioactive purified glycoproteins (GPI, GPII and GPIII) of baboon lung (Figs. 6b and 6c). GPI and GPII had a high content of polar amino acids and contained higher amounts of hydroxyproline, glycine and proline than the glycoprotein from hamster with pI 4.1 and 5.8.

The non-radioactive peak that focused at pI 8.4 had a high hydroxyproline, proline and glycine content. Its tyrosine and lysine contents were low. The peak focused at pI 8.4 had the amino acid composition of collagen (Table 2).

Discussion

The above results demonstrate the heterogeneity of glycoproteins associated with the lung collagen-elastin matrix. This heterogeneity exists in all three

Fig. 5. Gel filtration on Sepharose 4B of the macromolecules extracted with 5M-guanidinium chloride (A_1) from baboon (a) and from hamster (b) lung

Samples were reduced with dithiothreitol, alkylated with iodoacetamide and dialysed against 6M-urea/0.02M-EDTA buffer, pH 7.4, containing 0.2% SDS, before chromatography. The elution positions of standards are indicated as follows: 1 and 2, albumin; 3, myoglobin. V_0 was determined with Blue Dextran 2000. The ^{14}C -labelled fractions, which were investigated by sucrose-gradient isoelectric focusing, are designated by braces (—). ●, A_{280} ; ○, ^{14}C glucosamine radioactivity.

species investigated; baboon lung was chosen because of the similarity of its connective tissue to that of human lung (Collins & Jones, 1978); rat and hamster lung were chosen because they are currently used as experimental models for various pathologies.

Fig. 6. Sucrose-gradient isoelectric focusing of ^{14}C -glucosamine-labelled fractions eluted by exclusion chromatography

(a) Fraction from hamster lung; (b) fraction I from baboon lung; (c) fraction II from baboon lung. ^{14}C glucosamine-labelled fractions were concentrated on a Diaflo membrane and then dialysed against 6M-urea containing Dowex X8 resin to remove SDS. The fractions were then investigated by preparative sucrose-gradient isoelectric focusing as described in the text, with a continuous recording of absorbance. Each fraction was assayed for radioactivity and pH measurement. —, A_{280} ; ●, ^{14}C glucosamine radioactivity; ○, pH.

As the solubility of glycoproteins is not in itself a sufficient criterion to distinguish the circulating from the structural glycoproteins, the macromolecular incorporation of ^{14}C glucosamine was investigated. The results indicate that the pulmonary cells synthesize ^{14}C glucosamine-radiolabelled glycoproteins in the surviving lung tissue. In order to avoid a non-specific degradation of glycoproteins remaining in the insoluble defatted and desalted lung residue, they were extracted by 5M-guanidinium

Table 2. *Amino acid composition of lung glycoproteins separated by sucrose-gradient isoelectric focusing*
 Three preparations obtained by other authors are given for comparison: ^a Darnule *et al.* (1977); ^b Francis & Thomas (1975); ^c Sahu & Lynn (1979).

	Content (residues per 1000 amino acid residues)								
	Glycoproteins of baboon lung			Hamster lung		Non-radio-active peak	Lung micro-fibrils ^a	Acidic structural lung glycoproteins ^b	Alveolar glycoproteins ^c
	pI ...	GP I	GP II	GP III	4.1	5.8			
Hydroxyproline		19	18	5	0	0	115	6	10
Aspartic acid		83	79	97	82	116	70	95	105
Threonine		53	54	62	65	56	20	41	50
Serine		73	56	77	79	71	39	78	61
Glutamic acid		125	103	118	114	99	84	114	137
Proline		68	72	50	42	47	75	47	54
Glycine		133	130	83	99	80	273	90	113
Alanine		74	82	71	91	78	106	72	74
Carboxymethylcystine		tr*	6	4	tr	tr	0	24	14
Valine		53	59	60	76	70	34	58	64
Methionine		0	11	2	tr	tr	0	16	15
Isoleucine		39	48	52	62	66	20	49	44
Leucine		82	77	100	109	108	42	101	95
Tyrosine		31	23	34	19	21	tr	34	17
Phenylalanine		48	46	57	32	32	33	40	30
Histidine		41	26	13	26	19	27	19	20
Lysine		30	47	60	49	67	8	61	47
Arginine		62	63	36	49	56	36	48	56

* Abbreviation used: tr, trace.

chloride buffer before hydrolysis of collagen (A₁ extracts). They exhibited the highest incorporation of [¹⁴C]glucosamine during the 5 h incubation period. The failure to detect more than traces of uronic acid in the extract and the correlation between the bands stained by periodic acid/Schiff reagent and the radioactivity incorporation pattern observed on SDS/polyacrylamide-gel electrophoresis suggests that the guanidine-extracted glycoconjugates are glycoproteins rather than proteoglycans.

The glycoprotein extracts from baboon lung were characterized by their apparent molecular weight, isoelectric point and amino acid composition. GP I and GP II have amino acid compositions comparable with those of previously described lung glycoproteins (Table 2) (Sahu & Lynn, 1979; Darnule *et al.*, 1977).

The presence of hydroxyproline may indicate a contamination with collagen or the presence of collagen-type sequences associated with the glycoproteins, as was recently reported by Knight *et al.* (1980) for a glycoprotein component of the micro-fibrils isolated from bovine ligamentum nuchae. Isoelectric focusing on polyacrylamide gel of A₁ extracts demonstrated that [¹⁴C]glucosamine was predominantly incorporated into a component with a pI of about 7.5. The discrepancies between this pI

and the pI 5.4 obtained from sucrose-gradient isoelectric focusing suggests the possibility of contamination by collagen, also supported by the amino acid composition of the non-radiolabelled peak focused at pI 8.4 in sucrose gradient that is similar to that of collagen.

The amino acid composition of GP III (35000 daltons) is comparable with that of the lung glycosylated polypeptides previously extracted by Darnule *et al.* (1977) (Table 2). This 35000-dalton glycoprotein may well also be a subunit of an higher-molecular-weight glycoprotein, since it cannot be detected on SDS without previous reduction with dithiothreitol. A glycoprotein with a similar molecular weight was isolated from the aortic media of pig (Moczar *et al.*, 1977) and rabbit (Moczar *et al.*, 1979). The preparation obtained from pig aorta aggregated with acid-soluble calf skin collagen on isoelectric focusing in a sucrose gradient in 6 M-urea, suggesting an association between this glycoprotein and collagen.

Less than 3% of the total radioactivity incorporated could be extracted by seven sequential extractions with 5 M-guanidinium chloride after collagenase digestion (A₂ extract). They were not investigated in detail. The final residual radioactivity associated with elastin could only be solubilized after

digestion with elastase. It is possible that the A₂ extract represents microfibrillar components, as described also by Darnule *et al.* (1977).

The present study confirms the presence of several glycoproteins synthesized *in situ* by pulmonary cells and representing approx. 4% of the young adult lung weight, in all three species studied. Their differences in molecular weight, isoelectric point, amino acid and carbohydrate compositions appear to reflect their molecular heterogeneity.

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