

# Isolation and Characterization of Two Hydroxyproline-Containing Glycoproteins from Normal Animal Lung Lavage and Lamellar Bodies

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**ABSTRACT** Two glycopeptides, present in particulate material obtained by pulmonary lavage from normal rabbits, were isolated and characterized. The same two glycopeptides were present in preparations of lamellar bodies from rabbit lung. The estimated molecular weights of the two glycopeptides by sodium dodecyl sulfate-acrylamide gel electrophoresis were found to be 62,000 and 36,000, and both were found to contain hydroxyproline and relatively high amount of glycine (11 and 15%, respectively). Carbohydrate analysis of the two glycopeptides demonstrated the presence of glucosamine, sialic acid, mannose, fucose, and galactose. Similar glycopeptides of the same molecular weights, and amino acid and carbohydrate compositions have been found in lavage material isolated from lungs of patients with alveolar proteinosis. The data indicate that these two collagen-like glycopeptides are major intra-alveolar proteins in many mammals, including humans.

## INTRODUCTION

Alveolar proteinosis (1) is a chronic pulmonary disease of unknown pathogenesis in which the alveoli and terminal bronchioles of the lung are filled with a periodic acid-Schiff (PAS)<sup>1</sup> positive amorphous material. This material, insoluble in isotonic saline, can be removed in particulate form by pulmonary lavage (2, 3). Analysis of the saline-insoluble lavage material by sodium dodecyl

This work was presented in part at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April, 1973.

Received for publication 15 August 1974 and in revised form 2 December 1974.

<sup>1</sup>Abbreviations used in this paper: PAS, periodic acid-Schiff; SDS, sodium dodecyl sulfate.

sulfate (SDS)-polyacrylamide gel electrophoresis revealed the presence of three major peptides, the mol wt of which were 69,000, 62,000, and 36,000 (4). The latter two peptides were PAS-positive. Amino acid and carbohydrate analysis of the 62,000 and 36,000 mol wt peptides revealed the presence of hydroxyproline, hydroxylysine, glucose, and relatively large amounts of glycine (10 and 15%, respectively). The cause of excessive accumulation of these glycopeptides within alveoli of patients is unknown.

Complete characterization of the particulate proteins of lung lavage material from normal animals has not been previously reported. However, King, Klass, Gikas, Ruch, and Clements (5, 6) have identified several peptides in surface-active material isolated from lung lavage fluid of sheep and dogs. These authors found that these surface-active materials had the surface properties predicted for the surfactant lining the alveoli *in situ* (7).

It has been postulated (8-11) that lamellar bodies (osmiophilic concentric laminar organelles), present in type II epithelial cells of lung, are the storage or secretory form of the surfactant system. It has also been shown that the lamellar bodies and the surfactant activity appear simultaneously in the prenatal lung (12). One component of this surfactant system was shown to be a saturated phospholipid, dipalmitoyl phosphatidylcholine (13, 14), also found in lamellar bodies (15). The protein components of the lamellar bodies and the acellular lining fraction of the lung were found to contain mostly an albumin-like protein (15).

In this study, we present compositional data on two glycopeptides isolated from lung lavage material of normal rabbit lung and lamellar bodies that are very similar to the unique glycopeptides isolated from lungs of patients with alveolar proteinosis (4). The function of

these glycopeptides is not known, but that they occur as major alveolar glycopeptides in many species and accumulate excessively within alveoli of at least one species (human alveolar proteinosis) suggests that the peptides are alveolar secretions, probably derived from lamellar bodies of type II epithelial cells.

## METHODS

**Material.** Electrophoresis grade acrylamide, bisacrylamide, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine and especially pure Bio-Gel P-200 were obtained from Bio-Rad Laboratories, Richmond, Calif. SDS was purchased from Fisher Scientific Co., Pittsburgh, Pa. *p*-Hydroxybenzoic acid hydrazide was purchased from Aldrich Chemical Co., Milwaukee, Wis. The ampholyte was purchased from LKB Laboratorie och Kemikaliska Produkter, Stockholm, Sweden. Dansyl chloride was purchased from Pierce Chemical Co., Rockford, Ill.

**Preparation of lavage material.** Normal rabbits (7-8 lbs) were killed by air embolus, and the lungs were lavaged through the trachea, first with 150 ml (50 ml three times) of ice-cold isotonic saline and then with 150 ml (50 ml three times) of cold hypotonic EDTA (1 mM EDTA in 5 mM Tris-HCl buffer, pH 7.5). The saline lavage removes all the lipid. Very little protein was found in this lavage. To increase the amount of protein and glycoprotein, an EDTA wash was necessary. The isotonic fluid was discarded and hypotonic lavage fluid (EDTA wash) was centrifuged in the cold at 750 *g* for 10 min to remove alveolar macrophages and other cells. The supernate was then centrifuged in the cold at 25,000 *g* for 15 min. The precipitate, in particulate form, obtained by this centrifugation was suspended in 5 mM Tris-HCl buffer, containing 0.25 M sucrose and 1 mM EDTA (pH 7.5), and homogenized. To remove completely any remaining cells and alveolar organelles, the homogenized material was layered on 30% sucrose (wt/wt) and centrifuged at 4°C at 75,000 *g* for 1 h. The material at the interface was removed, suspended in 5 mM Tris-HCl buffer containing 1 mM EDTA (pH 7.5), and centrifuged at 100,000 *g* for 30 min. The precipitate was collected and washed five times with the same buffer. No cells or cellular organelles were present, as observed by electron microscopy. The appearance was that of inhomogeneous vesicles and membranous material.

**Isolation of lamellar bodies.** Lamellar bodies were isolated from rabbit lungs on a sucrose density gradient as described by DiAugustine (15). The final preparation contained 92% lipid and 8% protein. The white pellets obtained were free of other cell organelles, as determined by electron microscopy, and were stored under N<sub>2</sub> at -20°C before extraction of glycopeptides. The lipid content of this preparation was reported previously (15).

**Analysis.** Total protein was determined by the Lowry, Rosebrough, Farr, and Randall method (16), total carbohydrate by the phenol-sulfuric acid method (17) and sialic acid by the thiobarbituric acid method (18). Total lipid was estimated gravimetrically after three extractions with chloroform-methanol (2:1 vol/vol) of dry material.

SDS-acrylamide gel electrophoresis was performed according to the method of Weber and Osborn (19). The gels were either stained for protein with coomassie blue or for glycoproteins with PAS reagent (20). Molecular weight estimations for peptides on gels were made with bovine serum albumin, ovalbumin, lactate dehydrogenase, concanavalin A, and cytochrome *c* as standards.

After chloroform-methanol extractions, the proteins were solubilized in 5 mM Tris-HCl buffer containing 0.1% SDS and 0.05% 2-mercaptoethanol and applied to a Bio-Gel P-200 column (2.5 × 80 cm) equilibrated with the same buffer. Elution of peptides was done by the equilibrating buffer. The major peaks were checked for purity by SDS-gel electrophoresis.

**Amino acid and carbohydrate analyses.** Amino acid and amino sugar analyses were performed by the method of Spackman (21) by a Beckman 120B analyzer (Beckman Instruments, Inc., Fullerton, Calif.) on fractions eluted from the Bio-Gel column, as well as on stained and unstained bands cut from SDS-gels (22). The peptides were hydrolyzed in 6 N HCl in an evacuated, sealed tube at 110°C for 24 h. The excess gel material was removed by centrifugation when gel bands were used for hydrolysis. Analyses were also done by hydrolyzing the peptide eluted from gels by method of Weiner, Platt, and Weber (23). Recovery of known protein (bovine serum albumin or cytochrome *c*) from gels was over 80%. Possible contamination of acrylamide gels with amino acids was checked with blank gels as controls, under the same conditions as above. No impurities could be found. In addition, known proteins (bovine serum albumin, ovalbumin) were eluted from similar gels and analyzed for amino acids. The analyses were identical to those of the untreated starting proteins. Analyses of the peptides isolated from gels were similar to analyses of fractions obtained from Bio-Gel P-200 column. No corrections were made for the destruction or incomplete hydrolysis of the peptides under these conditions. Hydroxylysine and hydroxyproline analyses were done by the method of Piez and Morris (24) and also with a single column (Durrum DC-1A, Durrum Instrument Corp., Palo Alto, Calif.) and temperature gradient. The first 30 min of elution of amino acids was at 30°C. The temperature was then increased (over the next 15 min) to 60°C. Both hydroxy-amino acids were well separated on this column. Hydroxylysine and hydroxyproline analyses were also performed by the colorimetric method (25, 26). The content of these amino acids were similar, whether analyzed in the analyzer or by the colorimetric method. Neutral sugars were analyzed by the method of Lee, Johnson, White, and Scocca (27) after the peptides were hydrolyzed with 2 N trifluoroacetic acid for 3 h at 110°C. In addition, glucose was measured with glucose oxidase method as described before (4). Amino sugars were also estimated on the single column of amino acid analyzer after the peptide was hydrolyzed in 4 N HCl for 6 h at 100°C, as described by Spiro (28). Neutral sugars were also estimated by this method (28). The amounts of various sugars in these glycopeptides estimated by these different methods were the same.

**End-group analyses.** Qualitative identification of NH<sub>2</sub>-terminal amino acid residue of the 36,000-mol wt glycopeptide isolated from Bio-Gel column was performed by the dansyl procedure, as described by Gray (29) with the modifications suggested by Hartley (30). The NH<sub>2</sub>-terminal amino acid residues of the 62,000 and 36,000 mol wt glycopeptides eluted from SDS-acrylamide gels were also determined by the method of Weiner et al. (23). Both methods gave the same single NH<sub>2</sub>-terminal residue.

**Isoelectric focusing.** Isoelectric focusing of the 36,000 mol wt glycopeptide in polyacrylamide gels containing 3.5% acrylamide and in presence of 8 M urea was done by the method of Miller and Elgin (31). pH 3.5-10 ampholine was used. The pH gradient obtained was determined by the method of Finlayson (32). The glycopeptide was dia-

lyzed against 10 M urea containing 1% 2-mercaptoethanol for 12 h (one change) before electrofusing.

## RESULTS

Between 2 and 3 mg of lavage precipitate were obtained from the lungs of each rabbit. After purification on sucrose, about 40% of the total weight of each lavage remained.

It contained 56% lipid, as estimated after extraction with chloroform-methanol. SDS-acrylamide gel electrophoresis of the delipidated lavage material (Fig. 1C) revealed six major bands of apparent mol wt 75,000, 69,000, 62,000, 50,000, 36,000, and 25,000. The PAS staining of these gels (Fig. 1D) indicated two prominent glycopeptides of mol wt 62,000 and 36,000. Several other glycopeptides were also present in this material, as indicated by the PAS reagent.

Between 0.5 and 1 mg of protein was obtained from the lung lamellar bodies of each rabbit. The phospholipid-to-protein ratio in this preparation was 12:1. SDS-acrylamide gel electrophoresis (Fig. 1A) of the delipidated lamellar bodies revealed six major bands of apparent mol wt 75,000, 69,000, 62,000, 50,000, 36,000, and 25,000, the most prominent of which was the 69,000 mol wt peptide. The 62,000 and 36,000 mol wt peptides were also PAS-positive (Fig. 1B). Other PAS-positive bands are also present.

The 36,000 mol wt glycopeptide from lung lavage material was separated by gel filtration on a Bio-Gel P-200 column, as seen in Fig. 2. The 62,000 mol wt glycopeptide could not be separated from the 69,000 mol wt peptide in this column. Accordingly, amino acid

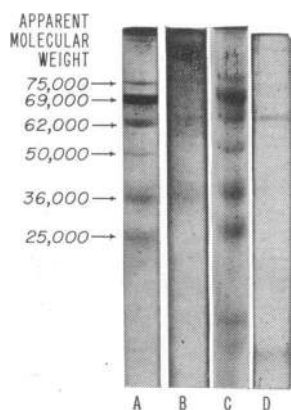


FIGURE 1 SDS-polyacrylamide gel electrophoresis of alveolar lavage sediment and lamellar bodies from rabbit lungs. Electrophoresis was performed with 10% acrylamide, containing approximately 60  $\mu$ g of protein in A, B, and C, and 40  $\mu$ g in D. A, Lamellar body preparation, stained with coomassie blue; B, Same as A, stained with PAS; C, Alveolar lavage sediment after sucrose density gradient centrifugation, stained with coomassie blue; and D, same as C, stained with PAS.

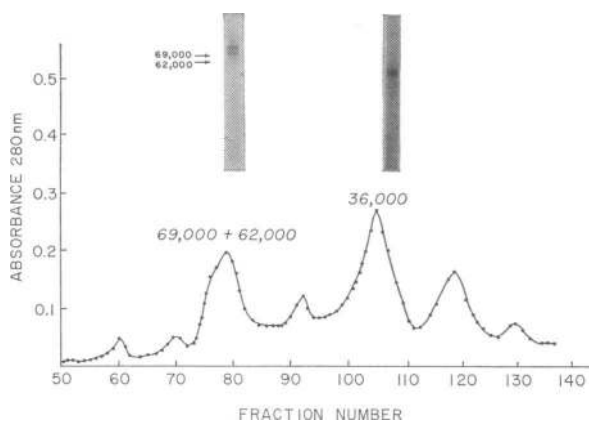


FIGURE 2 Separation of lung lavage peptides of rabbit by gel filtration on Bio-Gel P-200 column in 5 mM Tris-HCl buffer containing 0.1% SDS, 0.05% 2-mercaptoethanol, and 0.02% sodium azide (pH 7.5). Column dimensions, 2.5  $\times$  80 cm; volume per fraction, 0.8 ml. The peaks containing the purified peptides are shown after electrophoresis in SDS-polyacrylamide gels (insets). The electrophoresis was done by the method of Weber and Osborn (19).

analysis on the 62,000 mol wt glycopeptide was done on gel bands.

Separation of lamellar body peptides on a Bio-Gel P-200 column is given in Fig. 3. The 62,000 mol wt peptide could not be separated from the 69,000 mol wt peptide. Accordingly, the amino acid analysis of the 62,000 mol wt peptide was done on the gel band. The 36,000 mol wt peptide could easily be separated from other peptides on the Bio-Gel P-200 column, as seen in Fig. 3.

The calculated percentages of the 36,000 mol wt glycopeptide in lavage and lamellar bodies are approximately 32% and 20%, respectively. The percentage of the 62,000 mol wt peptide could not be calculated, since we have been unable to separate this glycopeptide from the 69,000 mol wt peptide except by gel electrophoresis.

The material of apparent very high molecular weight that failed to enter the gel (Fig. 1) is an artifact of loading. No high molecular weight protein is seen when the protein is dissolved in a larger volume of solubilizing medium and separated on Bio-Gel P-200 column (Figs. 2 and 3).

Preliminary amino acid sequence data and end-group analyses of cyanogen bromide cleavage fragments of the human 36,000 mol wt peptide<sup>a</sup> suggest that this peptide is homogeneous and contains 310 residues.

Isoelectric focusing (Fig. 4) of the reduced peptide (36,000) from lung lavage and lamellar bodies after 12 h of dialysis against 8 M urea failed to effect any further resolution. Only one area of protein, pH 7.4,

<sup>a</sup> Bhattacharyya, S. N., S. Sahu, and W. S. Lynn. Unpublished observation.

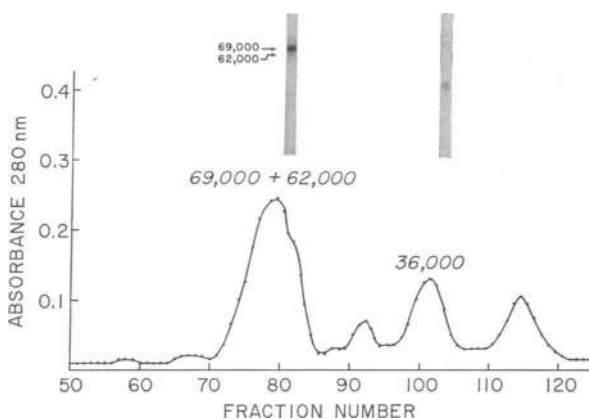


FIGURE 3 Separation of rabbit lung lamellar body peptides by gel titration on Bio-Gel P-200 column in 5 mM Tris-HCl buffer containing 0.1% SDS, 0.05% 2-mercaptoethanol, and 0.02% sodium azide (pH 7.5). Column dimensions, 2.5 × 80 cm; volume per fraction, 0.8 ml. Electrophoresis patterns of the peaks of the purified peptides in the SDS-polyacrylamide gel are shown in the insets. The gel electrophoresis was done by the method of Weber and Osborn (19).

was present. The data from electrofocusing suggest that the peptide is pure.

Amino terminal end-group analysis of the 62,000 and 36,000 mol wt glycopeptides (Table I) isolated from

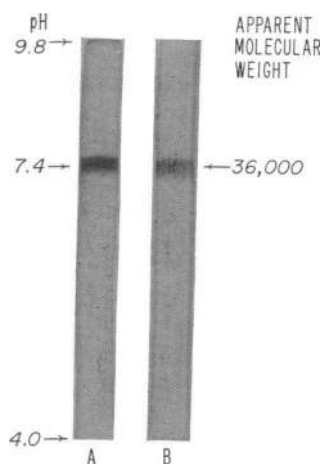


FIGURE 4 Isoelectric focusing of the 36,000 mol wt glycopeptide isolated from lavage (A) and lamellar bodies (B) of rabbit lung. Electrophoresis was performed with 3.5% acrylamide containing 8 M urea and 0.4% ampholine (pH 3.5–10.0), by the method of Miller and Elgin (31). Anode at bottom. Direction of migration top to bottom. The glycopeptide was dialyzed against 10 M urea containing 1% 2-mercaptoethanol for 12 h (one change) before electrofocusing. Samples were applied directly to the top of polymerized gel; a constant current of 1 mA/gel was maintained until a voltage of 200 V had been reached. A constant voltage of 200 was then maintained until the end of the run (6 h total time). About 60  $\mu$ g of protein was applied to gel A and 45  $\mu$ g of protein to gel B.

TABLE I  
Amino Acid Composition of Purified Glycopeptides  
from Rabbit Lung

Amino acid	Lavage glycopeptides		Lamellar body glycopeptides	
	62,000	36,000	62,000	36,000
Aspartic acid	96.0	81.0	95.8	80.9
Glutamic acid	115.9	90.5	116.0	90.5
Threonine	51.7	47.6	51.6	47.4
Serine	48.2	48.6	48.4	48.7
Proline	53.6	62.0	53.5	62.0
Glycine	110.0	148.8	110.0	148.6
Alanine	76.2	71.0	76.1	71.1
Half-cystine	12.4	17.4	12.3	17.6
Valine	63.5	65.6	63.5	65.8
Methionine	14.0	10.6	14.1	10.6
Isoleucine	29.6	44.5	29.5	44.6
Leucine	86.0	96.4	86.1	96.6
Tyrosine	46.7	33.8	46.7	33.8
Phenylalanine	50.2	41.8	50.1	41.9
Lysine	63.0	42.5	63.1	42.5
Histidine	24.8	23.6	25.0	23.5
Arginine	50.3	58.4	50.6	58.4
4-Hydroxyproline	8.2	12.0	8.3	11.9
Hydroxylysine	0	0	0	0
NH <sub>2</sub> -terminus	Valine	Threonine	Valine	Threonine

Amino acid compositions are expressed as residues/1,000 amino acids. The results are average of triplicate analyses of 24-h hydrolysates; no corrections have been made for destruction or incomplete hydrolysis.

presence of only one NH<sub>2</sub>-terminal residue for each human or rabbit lavage or lamellar bodies indicated the glycopeptide, i.e., valine and threonine, respectively. These data also indicate that these glycopeptides are probably pure.

Amino acid compositions of the two glycopeptides (62,000 and 36,000 mol wt) from lavage material and lamellar bodies of rabbit lungs are given in Table I. Both glycopeptides contain the same content of hydroxyproline as well as other amino acids, whether isolated from lavage material or lamellar bodies. Hydroxylysine was not found in the glycopeptides isolated from lung lavage material or from lamellar bodies.

There is also a major band of mol wt 69,000 in gels of lavage material and in lamellar body preparations. This peptide contained no carbohydrate, and had an amino acid composition similar but not identical to rabbit albumin from commercial sources. The content of glycine was high, while the content of valine was low in comparison to the rabbit albumin. That the high glycine content is not an artifact contributed by acrylamide gel was shown by analyzing pure rabbit, bovine, and human albumin, isolated electrophoretically on SDS-acrylamide gels. The glycine content of the authentic preparation was 1–2%, in good agreement with the known content of glycine in albumin (33). A similar observation (4) was made in the case of the 69,000 mol wt peptide isolated from gels of lung lavage material of patients with alveolar proteinosis. This peptide, iso-

TABLE II  
Carbohydrate Composition of Purified Glycopeptides  
from Rabbit Lung

Carbohydrate	Lavage glycopeptides		Lamellar body glycopeptides	
	62,000	36,000	62,000	36,000
	% carbohydrate/peptide			
Sialic acid	1.20	1.50	1.22	1.50
Mannose	1.50	2.00	1.50	1.95
Fucose	0.60	0.78	0.60	0.76
Galactose	1.00	2.20	1.00	2.20
Glucosamine	0.62	0.60	0.62	0.60

The compositions are expressed as percentage of carbohydrate per peptide. The methods of analysis are given in the text. Results are average of triplicate analyses.

lated from human lavage precipitate, cross-reacts with rabbit antisera to human albumin (4).

The carbohydrate compositions (Table II) of the glycopeptides (62,000 and 36,000 mol wt) from lavage material and lamellar bodies of rabbit lungs indicated the presence of sialic acid, glucosamine, mannose, fucose, and galactose in amounts essentially the same as in the glycopeptides isolated from human lungs with alveolar proteinosis, except that glucose was not present in either of the rabbit lung glycopeptides. Although glucose was originally reported in the human glycopeptides, subsequent studies have indicated that the glucose was bound to contaminating glycolipids.<sup>3</sup>

## DISCUSSION

It has been previously reported (4) that the lavage material isolated from lungs of patients with alveolar proteinosis contained two unique glycopeptides of apparent mol wt 62,000 and 36,000. These glycopeptides, insoluble in saline, were associated with lipid (52% by weight) and contained hydroxyproline and high amount of glycine.

The results presented in this paper show that the lavage material and the lamellar bodies from normal rabbit lungs also contain two glycopeptides of the same compositions, NH<sub>2</sub>-terminal amino acid residues, and apparent mol wt (62,000 and 36,000) as those found in human lung. Large amounts of lipids are also associated with lung lavage material (56% by weight) and lamellar bodies (90% by weight) from rabbits. Preliminary results<sup>4</sup> on dog lung lavage material also indicated the presence of two glycopeptides with similar compositions.

The SDS-acrylamide gel patterns, isoelectric focusing, and the qualitative NH<sub>2</sub>-terminal amino acid analy-

ses suggest that these glycopeptides are homogeneous. The estimation of molecular weight of the glycopeptides by SDS-acrylamide gel electrophoresis as given here is, however, probably not sufficiently accurate. This is because the carbohydrate and collagen-like portions<sup>2</sup> of the molecules may influence the rate at which the glycopeptides would migrate through acrylamide gels in SDS. The presence of carbohydrate has led to the overestimation of molecular weights of immunoglobulin chains (34, 35). Also collagens and peptides derived from them migrate through acrylamide gels in SDS at a much slower rate than globular proteins of the same molecular weight (35, 36). The overestimation of molecular weights of the subunits of human C1q was also noted by Reid, Lowe, and Porter (37).

It was previously reported (4) that hydroxylysine and glucose were present in the glycopeptides isolated from lavage material of patients with alveolar proteinosis. After careful delipidation of the lavage material from human (alveolar proteinosis) and animal lungs, all the glucose was found to be in two glycolipids<sup>3</sup> (glucosyl ceramide and the ganglioside, GM<sub>3</sub>). The reported presence of small amounts of hydroxylysine in the glycopeptides from lungs of patients with alveolar proteinosis was an artifact, because the peak indicating hydroxylysine in the amino acid analysis disappeared upon extensive delipidation and further purification of the peptides by Bio-Gel P-200 column. The absence of hydroxylysine in these glycopeptides was also verified by the colorimetric method (25).

The presence of these unique glycopeptides as major peptides in lung lavage material and lamellar bodies in particulate form suggests that the lamellar bodies may be the source of these glycopeptides. The actual biochemical and physiological functions of the lamellar bodies in type II epithelial cells of lungs is not known. Sorokin (38) has reported transformation of the type II cell multivesicular bodies into type II cell lamellar bodies. The presence of multivesicular bodies in the lamellar body fractions during purification procedures also lends support to such a transformation or fusion (15). The mature organelle appears to migrate to the apical area of the type II epithelial cells, where it is secreted.

King et al. (5, 6) have described a soluble peptide of mol wt 34,000 in the material lavaged from different animal lungs, but the compositional data on this peptide are not yet available. These authors concluded that this peptide was present in the surface-active material. Since the method of isolation of lavage material in this study is very similar to that of King et al. (5), their peptide may be the same as the 36,000 mol wt glycopeptide reported here.

The presence of hydroxyproline and a relatively high content of glycine in the 62,000 and 36,000 mol wt

<sup>3</sup>Sahu, S., and W. S. Lynn. Unpublished data.

<sup>4</sup>Bhattacharyya, S. N., M. A. Passero, and W. S. Lynn. Unpublished observation.

glycopeptides suggests contamination of these peptides by collagen. But these glycopeptides do not contain hydroxylysine and glucose, usually found in collagens or collagen-related proteins of basement membrane (39-41). Moreover, the percentages of hydroxyproline and glycine in these glycopeptides are much lower than in collagen. It is also interesting to note that neither of the glycopeptides contain 3-hydroxyproline, known to be present in the basement membrane (40). The ratio of glucosamine to galactosamine in most basement membrane preparation is 4:1 (40), whereas in the alveolar glycopeptides, no galactosamine was found.

The existence of collagenous and noncollagenous sequences in the same polypeptide chain is now well-documented for procollagen (42, 43). Skoza and Mohos (44) have recently reported that glomerular basement membrane preparations consist of two glycoproteins, collagen and sialoprotein. Reid (45) has also shown that one of the subunits of human complement, C1q, contains a short collagen-like sequence, (Gly-X-Y)<sub>n</sub>, in which 5 of 26 of the Y residues are hydroxyproline. In addition, C1q contains hydroxylysine and glucose (45-47). Since the alveolar peptides contain no hydroxylysine or glucose, it is likely that they are not derived from C1q (46). The alveolar glycopeptides are, therefore, another example of peptides containing hydroxyproline and high concentration of glycine, and which, like procollagen (42, 43) and C1q (45), may contain collagen-like sequences of (Gly-X-Y)<sub>n</sub> in one segment of the peptide chain but not in other segments.

As shown by this report, two unique collagen-like glycopeptides are present in particulate form in the lung lavage material and lamellar bodies isolated from normal rabbit. We suggest that these glycopeptides are normal components of alveoli and probably are normal secretory products of type II epithelial cells. That in humans with alveolar proteinosis these glycopeptides accumulate without producing any inflammatory cellular response also suggests that these peptides are normal components of alveoli.

#### ACKNOWLEDGMENTS

This investigation was supported in part by National Institutes of Sciences Training Grant ES-00124-06, Veteran's Administration Project 5348-01, and a North Carolina Lung Association Grant.

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