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Structural features of human tracheobronchial mucus glycoprotein

Mary C. ROSE,*§ William A. VOTER,† Charles F. BROWN* and Bernard KAUFMAN‡
** Department of Medicine, † Department of Anatomy and ‡ Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, U.S.A.*

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Electron microscopy of platinum-shadowed preparations of human tracheobronchial mucins showed very flexible filamentous structures that frequently occurred in an intricate random-coiled pattern of filament(s) surrounding a dense core-like domain. The filament(s) associated with cores accounted for 70–80% of the mass of the mucin preparation, the remainder being accounted for by free filaments. On aggregation, the molecules formed a large interwoven network quite different from the massive rope-like structures characteristic of sheep submaxillary mucin aggregates [Rose, Voter, Sage, Brown & Kaufman (1984) *J. Biol. Chem.* **259**, 3167–3172]. Mild sonication resulted in extensive fragmentation of the tracheobronchial mucin molecules and yielded short filaments of various lengths, free cores and some cores associated with short filaments. Mucin glycopeptide fragments obtained by proteolytic digestion were flexible, core-free, filaments. The glycopeptides obtained by Pronase digestion were shorter than those obtained by tryptic digestion. The intricate structures of human tracheobronchial mucin differ markedly from the extended filaments reported for sheep submaxillary and human ovarian-cyst mucins but agree with the roughly spherical expanded model proposed for mucins by Creeth & Knight [(1967) *Biochem. J.* **105**, 1135–1145] on the basis of hydrodynamic measurements.

Mucins are a class of glycoproteins that are secreted by certain types of epithelial cells localized on the luminal surface of various ductular and tubular structures. These glycoproteins characteristically contain several hundred oligosaccharide chains in *O*-glycosidic linkages to serine and threonine residues of the polypeptide chain and are rich in serine, threonine, proline and glycine, which account for 50–85% of the total amino acid residues (Gottschalk, 1972). Although all mucins share these general features, individual types differ in complexity with regard to their saccharide chains, polypeptide backbone and physical properties. Of the mucins examined to date, sheep submaxillary mucin is the simplest and best characterized (Gottschalk *et al.*, 1972; Hill *et al.*, 1977); human tracheobronchial mucins are more complex.

Interactions between mucin molecules yield high-molecular-mass oligomers ($M_r \geqslant 10^6$), as demonstrated both by ultracentrifugation (see Gottschalk *et al.*, 1972, for review; Hill *et al.*, 1977; Feldhoff *et al.*, 1979) and by molecular-sieve chromatography (Rose *et al.*, 1979*a*,*b*). In the case of sheep submaxillary mucin, increasing the mucin concentration beyond that resulting in aggregation leads to an increase in viscosity, whereas with the more complex mucins (bovine cervical, pig gastric and human tracheobronchial mucins) it leads to the separation of a viscoelastic gel phase (Gibbons & Sellwood, 1973; Allen *et al.*, 1976; Rose *et al.*, 1979*a*).

Recently we have examined sheep submaxillary mucin by heavy-metal shadowing and have shown that it has the appearance of an extended filament and assumes a globular shape on removal of the oligosaccharide chains (Rose *et al.*, 1984). In the present paper on the structural features of human mucins we demonstrate that tracheobronchial mucins are very intricate molecules with an overall architecture consistent with the flexible, roughly spherical, model proposed for ovarian-cyst-fluid mucin (Creeth & Knight, 1967) and subsequently extended to bronchial mucins (Harding *et al.*, 1983).

§ Present address: Department of Child Health and Development and Department of Biochemistry, George Washington University, Children's Hospital, 111 Michigan Avenue N.W., Washington, DC 20010, U.S.A.

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Experimental procedures

Sample preparation

Human tracheobronchial mucin from patients with bronchial asthma was isolated as previously described (Rose *et al.*, 1979*a*). Mucin from normal individuals and patients with cystic fibrosis were purified by the same procedures (M. C. Rose, C. F. Brown, J. Z. Jacoby, W. S. Lynn & B. Kaufman, unpublished work). Mucin samples purified by CsBr-density-gradient centrifugation (Woodward *et al.*, 1982) were kindly provided by Dr. Eugene Davidson (University of Pennsylvania, Hershey, PA, U.S.A.). Sonicated samples were prepared in a Branson S-125 Sonifier with a microprobe tip. The sample containing mucin (1 mg/ml in 0.15M-ammonium acetate) was immersed in an ice bath and subjected to two 10s bursts of ultrasound at low frequency separated by a 15s cooling period.

Proteolytic digestion

Mucin glycopeptide fragments were prepared by digestion with trypsin and with Pronase and isolated by chromatography. For tryptic digestion, mucin was dissolved in 0.05M-ammonium bicarbonate buffer, pH7.6, at a concentration of 5mg/ml and digested with 5μl (100μg/ml in 1mM-HCl) of trypsin [1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated; Worthington Biochemical Corp.] at 37°C for 48h. At 24h additional enzyme (5μl) was added. The enzyme was destroyed by boiling; sufficient sodium dodecyl sulphate and ammonium acetate were added to adjust the sample to the concentration of the eluent buffer (0.1% sodium dodecyl sulphate and 0.15M-ammonium acetate) and the sample was chromatographed on Sepharose 4B. The tryptic glycopeptide, as monitored by neutral hexose (Dubois *et al.*, 1956), was eluted with a K_d of 0.35. The appropriate fractions were pooled, dialysed extensively to remove sodium dodecyl sulphate (Rose *et al.*, 1979*a*) and freeze-dried.

For Pronase digestions, 2mg of mucin was dissolved in 1ml of Tris buffer (0.1M-Tris, pH8.0, containing 5mM-$CaCl_2$). After addition of 20μl of Pronase stock [5mg of Pronase (Calbiochem)/ml of Tris buffer] and a drop of toluene, the sample was incubated at 37°C for 72h. An additional 20μl of Pronase stock was added at 24 and 48h. The activity of the Pronase was measured with *N*-α-benzoyl-DL-arginine *p*-nitroanilide (Sigma Chemical Co.) as substrate; over each 24h period the Pronase lost 50% activity. After the 72h incubation, the digest was dialysed for 24h at 4°C against 0.01M-NaCl and then for 24h against distilled water. After dialysis, ammonium acetate was added to a final concentration of 0.01M and the sample was chromatographed on Sephadex G-50 with 0.1M-ammonium acetate, pH7.6, as eluent buffer. The fractions were monitored at 280nm and by the phenol/H_2SO_4 method (Dubois *et al.*, 1956). A control reaction mixture was prepared and incubated as described above and also chromatographed under identical conditions. The glycopeptide fraction from the mucin digest was eluted in the void-volume fraction. An included peak containing no carbohydrate was eluted at the same position as the only peak observed in the control incubation.

Electron microscopy

Shadowed specimens of mucins were prepared for electron microscopy as previously described (Rose *et al.*, 1984). Mucin samples were diluted into buffers containing 50% (v/v) glycerol and ammonium acetate (0.075 to 2M) to a final mucin concentration of 30–75μg/ml. Approx. 20μl of each solution was sprayed on to a freshly cleaved mica surface and vacuum-dried at room temperature in a Balzers BAE 120 high-vacuum coating unit (Lichtenstein). The specimens were rotary-shadowed with platinum/carbon at a grazing angle of 6.5–8°. Measurement of contour lengths was performed as previously described (Rose *et al.*, 1984). Widths were determined by measuring the thickness of the filaments and corrected for a 4nm thickness of platinum (Fowler & Erickson, 1979). However, the widths can only be considered an estimate, as correction factors are not always reliable (Tyler & Branton, 1980).

Results

Platinum-shadowed molecules of human tracheobronchial mucin displayed several prominent structural features (Fig. 1*a*). The most consistent structures were flexible filaments that exhibited frequent sharp bends and appeared to be folded in a very intricate pattern. A dense globular core region was consistently observed in these tangled filamentous structures. These structures formed aggregates that resembled masses of interwoven fibres (Fig. 1*b*).

The tangled filamentous core structures accounted for 70–80% of the total filament lengths observed in the micrographs. Widths of 2–3nm were estimated for the filaments. Lengths ranging from 550 to 1100nm were measured for several of the less-tangled filamentous structures containing cores. However, it was not possible to measure accurately the length of a sufficient number of

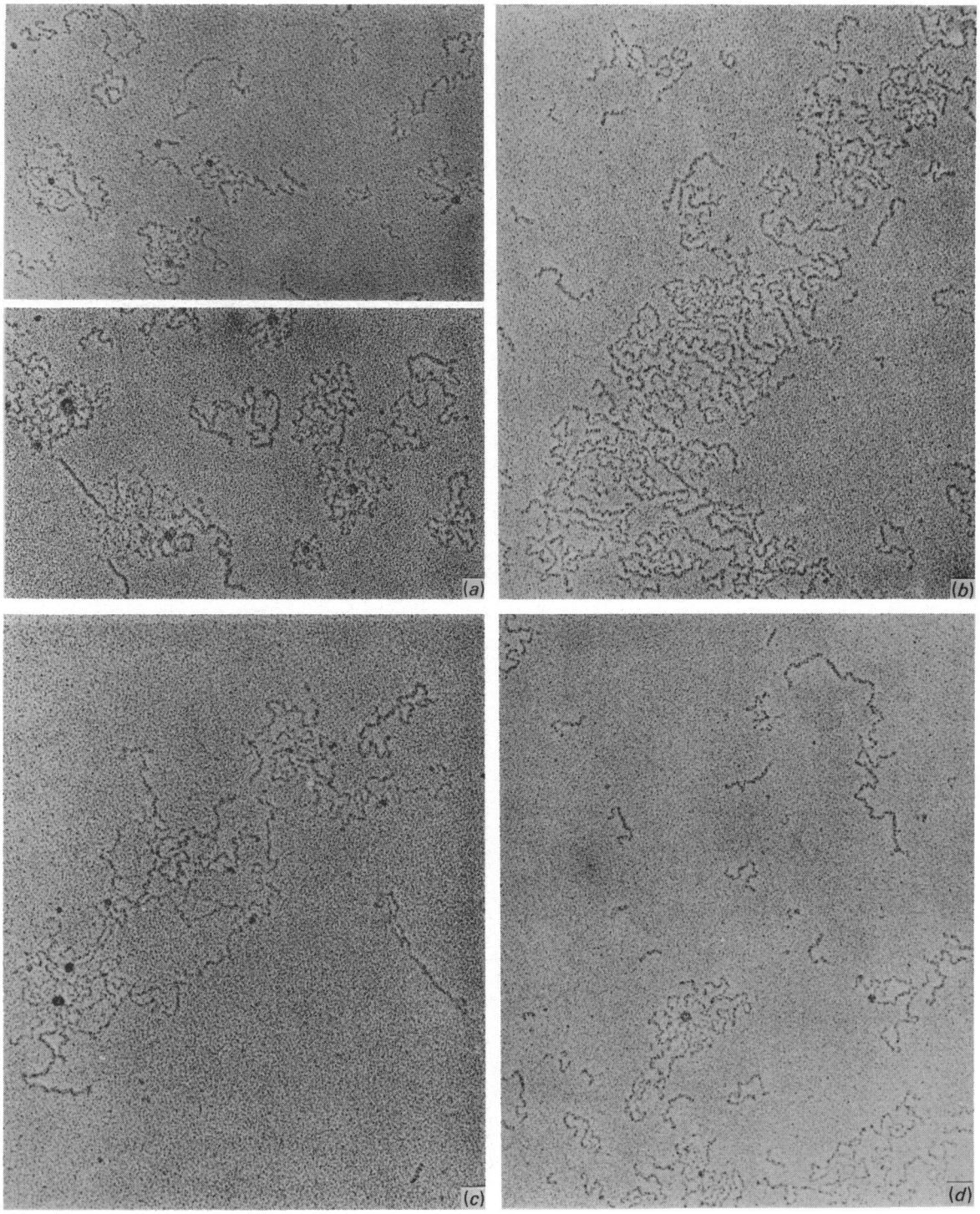

Fig. 1. *Electron micrographs of rotary-shadowed human tracheobronchial mucins*
All magnifications were ×90000. (*a*) Mucin from an individual with bronchial asthma. The mucin was purified by chromatography on Sepharose 4B and Sepharose 2B in the presence of reducing and dissociating agents. (*b*) Aggregates of bronchial-asthma mucin (*a* sample). (*c*) Unfractionated lung mucus sample, from which the mucin shadowed in (*a*) was purified. (*d*) Reduced carboxymethylated mucin from healthy respiratory airways.

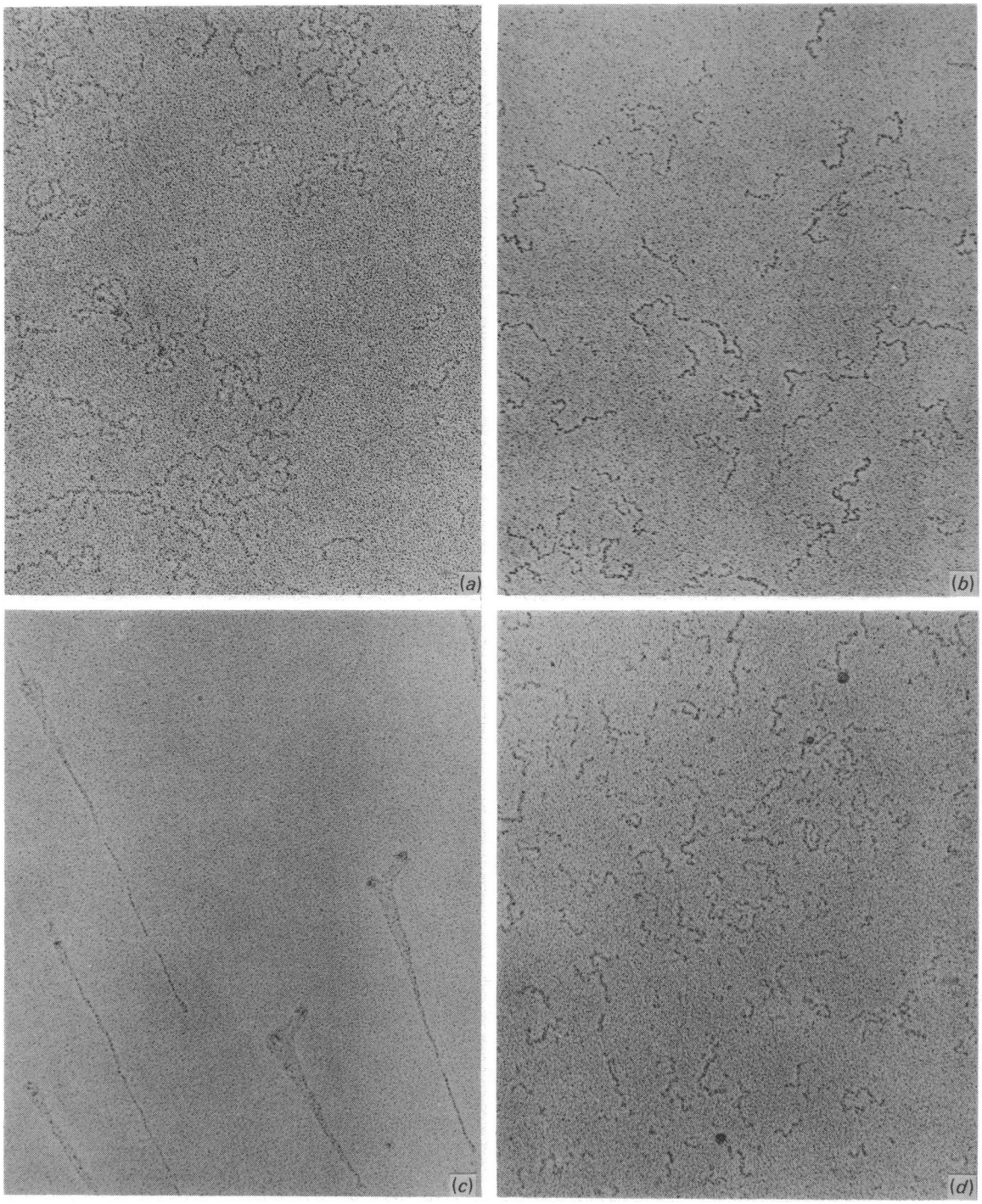

Fig. 2. *Electron micrographs of rotary-shadowed human tracheobronchial mucins*
All magnifications were ×90000. (*a*) Mucin prepared by CsBr-density-gradient centrifugation. (*b*) Glycopeptide fragments, obtained by tryptic digestion of mucin from an asthmatic (Fig. 1*a* sample). (*c*) Mucin (Fig. 1*a* sample) shadowed in the absence of glycerol. (*d*) Mucin (Fig. 1*a* sample) after mild sonication.

filaments to generate a histogram because of the non-linearity of the filaments and the uncertainty as to where the filaments began and ended. Filaments lacking dense globular core regions accounted for the remainder of the sample and may be fragments generated by shearing.

Molecules with features comparable with those of the bronchial-asthmatic mucins observed in Fig. 1(*a*) were also seen in micrographs of unfractionated lung mucus (Fig. 1*c*) and of normal (Fig. 1*d*) and cystic-fibrosis (micrograph not included) tracheobronchial mucins as well as with specimens of tracheobronchial mucin purified by CsBr-density-gradient centrifugation (Fig. 2*a*).

Tracheobronchial mucins that had been reduced and carboxymethylated (Fig. 1*d*) exhibited similar appearances to mucins that had been purified either in the presence (Fig. 1*a*) or in the absence (Fig. 2*a*) of reducing agents. The characteristics of mucin samples in dissociating solvents could not be assessed, as sodium dodecyl sulphate in excess of 0.1% interfered with the shadowing procedure and 5M-guanidininium chloride or 2M-CsCl solutions are too high in solute concentration to be useful for shadowing. Micrographs of samples (1mg of mucin/ml) dissolved in 0.1% sodium dodecyl sulphate and diluted 40-fold just before spraying showed the same profile of molecules as seen in Fig. 1(*a*).

In contrast, the glycopeptide fragment isolated from proteolytic digests of tracheobronchial mucins displayed only flexible filaments of various lengths (Fig. 3); neither intricate folded structures nor cores were observed (Fig. 2*b*). The glycopeptide molecules obtained by Pronase digestion (Fig. 3*b*) were shorter than those obtained by tryptic digestion (Fig. 3*a*). The estimated widths of the glycopeptide fragments were essentially the same as those of the intact mucin molecules.

The structural features described above were consistently observed in microscopy samples prepared by dissolving the mucin in 0.3M-ammonium acetate and diluting the preparation with an equal volume of glycerol to give a solution of 0.15M-ammonium acetate and 50% glycerol containing 30–75μg of mucin/ml. The glycerol concentration could be decreased 5-fold from 50% to 10% (decreasing the relative viscosity from approx. 6.67 to 1.32) without producing any observable changes in the shadowed images. However, when mucin samples containing no glycerol were sprayed and shadowed, bizarre-shaped structures, similar to those seen by Lamblin *et al.* (1979) and by Harding *et al.* (1983), were obtained (Fig. 2*c*). The problems in examining samples sprayed from glycerol-free solutions have been noted previously (Tyler & Branton, 1980) and are discussed below.

The effect of sonication on tracheobronchial mucin was examined because mucin prepared from sonicated bronchial washings (Sachdev *et al.*, 1980) has been reported to be much smaller (M_r 69400) than bronchial mucins characterized by Creeth *et al.* (1977), which have $\bar{M}_w^0$ values of 2×10^6–5×10^6. Electron micrographs of sonicated samples displayed globular cores, small flexible filaments with cores still attached, and numerous core-free small filaments of various lengths (Fig. 2*d*).

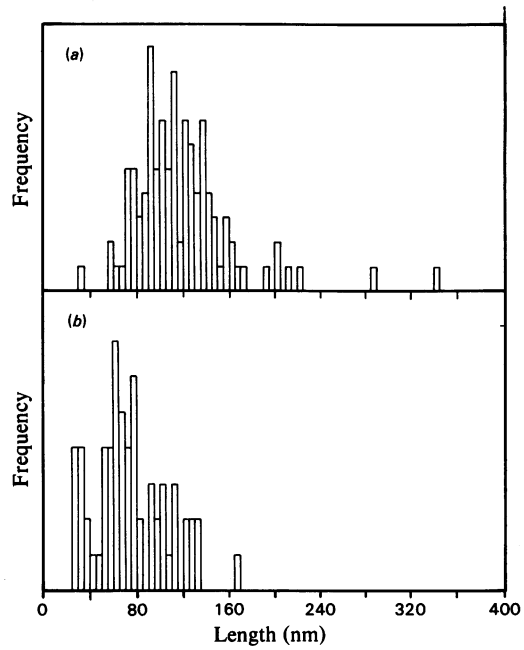

Fig. 3. *Histogram of the lengths of glycopeptide molecules obtained by proteolytic digestion of human tracheobronchial mucin*

(*a*) Tryptic-digest glycopeptide fragments. A total of 106 molecules were measured. The filaments had a number-average length of 120 ± 44nm. (*b*) Pronase-digest glycopeptide fragments. A total of 62 molecules were measured. The filaments had a number-average length of 75 ± 31nm.

Discussion

The intricate structures observed in tracheobronchial mucin samples purified by molecular sieving (Fig. 1*a*) and by density-gradient centrifugation (Fig. 2*a*) were also observed in unfractionated mucosal samples (Fig. 1*c*). These results indicate that the observed intricate forms of human tracheobronchial mucins were not the result of structural changes introduced by using reducing and dissociating solvents during purification.

The structures observed in Figs. 1(*a*), 1(*d*) and 2(*a*) were distinctly different from those reported for human bronchial mucins by Lamblin *et al.* (1979) and by Harding *et al.* (1983). The rotary-shadowed neutral mucin molecules in the micrographs shown by Lamblin *et al.* (1979) resemble flat sheet-like aggregates, and their acidic mucins exhibited some 'bizarre molecular forms' as well as some extended filaments. The bronchial mucins observed on unidirectional shadowing by Harding *et al.* (1983) were 'very flattened, with low profile areas barely resolved from background'; indeed, the latter images, as well as those of ours obtained in the absence of glycerol (Fig. 2*c*), appear quite similar to those reported for neutral bronchial mucins by Lamblin *et al.* (1979). The observed differences between the above-described micrographs and those shown in Figs. 1(*a*), 1(*d*) and 2(*a*) could reflect differences in sample preparation, as glycerol was absent from the sample solutions studied in the other two laboratories. Tyler & Branton (1980) studied the effect of glycerol on extended molecules and reported that structural details were not discernible and that only low-contrast images were obtained in the absence of glycerol, whereas samples that contained glycerol yielded images consistent with those obtained with other electron-microscopy procedures. Perhaps the interaction of some types of molecules with the mica (aluminium silicate) surface are minimized in the presence of glycerol.

Although the structures of human tracheobronchial mucins reported in the present paper differ markedly from those described by Harding *et al.* (1983), they are in general agreement with the flexible extended roughly-spherical model for ovarian-cyst fluid mucus glycoproteins proposed by Creeth & Knight (1967, 1968) on the basis of hydrodynamic measurements, a model subsequently extended to bronchial mucins by Harding *et al.* (1983). Similar structures for tracheobronchial mucins have also been observed via electron microscopy by Jenssen *et al.* (1980) after staining with phosphotungstic acid. Their tracheobronchial mucin specimens exhibit several variable and flexible structures that contain a central globular core; additional dense globular regions are also observed on the filamentous structures.

The intricate filamentous structures (Figs. 1*a*, 1*d* and 2*a*) may represent a single random-coiled polypeptide chain, which would have an expected diameter of 1–1.5nm if all the mucin oligosaccharide chains were mono- or di-saccharides (Rose *et al.*, 1984). The additional width presumably reflects the presence of larger oligosaccharide chains. A total analysis of the oligosaccharide chains of human tracheobronchial mucin (Roussel *et al.*, 1975) has not been completed, but di- to penta-saccharides constitute a significant component of the neutral oligosaccharide chains of CF bronchial mucin (Van Halbeek *et al.*, 1982).

The observed inner core-like globular structures of the mucin molecules may result from multiple cross-overs of the polypeptide chain. However, mild sonication of lung mucin did not result in loss of core structures, but rather fragmented the molecule and yielded core-free filaments, filaments with cores attached and cores (Fig. 2*d*). If covalent bonds of the polypeptide chain of viscoelastic mucins are fragmented by sonication, as are covalent bonds of DNA, then the observed filaments and the globular cores may represent two structural domains in the human tracheobronchial mucin molecule, perhaps the glycopeptide and naked peptide domains postulated for ovarian-cyst-fluid mucin (Donald, 1973) and for tracheobronchial mucin (Feldhoff *et al.*, 1979; Rose *et al.*, 1979*b*). It has been suggested, on the basis of electron-microscopy studies on an ovarian-cyst mucin sample presumed to be devoid of naked peptide regions, that the glycopeptide domains of mucins are represented by globular cores rather than by extended filaments (Harding *et al.*, 1983). However, extended filaments have been observed for ovarian-cyst (Slayter *et al.*, 1974) and sheep submaxillary mucin (Rose *et al.*, 1984) and for mucin-type glycoproteins such as epiglycanan (Slayter & Codington, 1973) and lubricin (Swann *et al.*, 1981). In addition, the glycoproteins obtained by proteolytic digestion of tracheobronchial mucin yielded flexible filaments (Fig. 2*b*) similar in structure to some of the molecular forms described by Lamblin *et al.* (1979) for acidic bronchial mucins. Since human bronchial mucins prepared by the procedure of Lamblin *et al.* (1979) have since been shown to be partially digested during purification (Houdret *et al.*, 1981), it is likely that some of the filamentous structures observed by Lamblin *et al.* (1979) represent mucin glycopeptide fragments.

An alternative explanation of the observed structures is that the filamentous and core structures are actually subunits of the tracheobronchial mucin molecule associated via some as yet undefined interactions. The number of filamentous subunits associated with a core-like region is indeterminate and varies from three to seven. If this explanation proves to be correct, then human tracheobronchial mucin has a different architecture from that of pig gastric mucin, which has been postulated to have four subunits attached to a globular domain via thiol interactions (Allen *et al.*, 1980). Finally, the core structures may represent domains in which lipids are non-covalently associated with tracheobronchial mucin (Bhaskar & Reid, 1981; Woodward *et al.*, 1982).

The aggregates formed by the tracheobronchial mucins resembled masses of interwoven fibres (Fig. 1*b*) analogous to those of agarose complexes (Arnott *et al.*, 1974; Laas, 1975), and were quite different from the massive rope-like structures characteristic of aggregates of sheep submaxillary mucin (Rose *et al.*, 1984). The expanded structures may be characteristic of mucins capable of forming viscoelastic gels, whereas the extended filamentous structures may reflect mucins, such as sheep submaxillary mucin, that are highly viscous and aggregate but do not form viscoelastic gels. The differences in the overall architecture of sheep submaxillary and human tracheobronchial mucins suggest the likelihood that more than one prototype for the structure of mucus glycoproteins will be required. Indeed, the expanded roughly spherical model proposed for ovarian-cyst mucin by Creeth & Knight (1967) and subsequently extended to bronchial mucins by Harding *et al.* (1983) is not representative of sheep submaxillary mucus glycoproteins. Since mucins from different species and organs have different biochemical components, oligosaccharide chains, polypeptide backbones and physical properties, it will not be surprising if gastric, intestinal and cervical mucins also have different overall structures. The relationship of structure to physiological function remains to be established.

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