The Separation and Characterization of Bronchial Glycoproteins by Density-Gradient Methods

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1. Sputum samples from a total of 18 asthmatic and chronic bronchitic patients were examined by analytical density-gradient ultracentrifugation. CsBr was used as the dispersal agent and dense electrolyte. 2. The patterns show two main groups of components, banding at about 1.3 g/ml and 1.5 g/ml; in addition, a few samples showed a further zone at approx. 1.65 g/ml. These components were identified as protein, secretory glycoprotein and DNA respectively. The glycoprotein zone was frequently hypersharp, and usually contained two or more partially resolved bands; it was always well resolved from the protein. 3. The glycoprotein components were isolated from nine representative sputum samples by density-gradient ultracentrifugation on a preparative scale. Analytical density-gradient ultracentrifugation was used to monitor the efficiency of the separations. 4. Some sputum samples separated cleanly under these conditions, the glycoprotein being essentially devoid of free protein; in others, separation was apparently incomplete, although computer simulation indicated that the conditions were adequate to ensure separation. Further density-gradient separations in CsCl were necessary with several samples before satisfactory products were obtained; mixtures of CsCl with guanidinium chloride were no more effective than CsCl alone. The reluctance to separate indicates a very strong, but non-covalent, interaction between protein and glycoprotein, probably associated with the gelatinous character of the secretion. 5. The purified glycoprotein components were characterized analytically and physicochemically. They contained *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, fucose and *N*-acetylneuraminic acid, and had an amino acid composition in which serine, threonine and proline predominated; however, aspartic acid, glutamic acid and cystine were also appreciable. The glycoproteins were of very high molecular weight, and usually showed more than one component in sedimentation velocity; their distribution in a density gradient indicated a substantial, but largely monotonic, density heterogeneity. 6. Thiol reduction decreased the molecular weight very substantially, but the products were relatively more homogeneous than the native materials. The amino acid composition was changed significantly and a small and variable proportion of protein or peptide was liberated. It is concluded that the native materials are disulphide-linked aggregates, probably through a cross-linking peptide, in confirmation of earlier studies.

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Normal bronchial secretion provides mechanical and immunological protection to the tracheo-bronchial epithelium (e.g. Dulfano, 1973); however, the normal secretion is scanty in volume and difficult to obtain (Schulze & Heremans, 1966). Accordingly, the copious secretions produced as sputum by some chronic bronchitic and asthmatic patients have been the main source of material for biochemical investigation (Brogan, 1959). However, investigations of the

properties of sputum are of interest in their own right, because both the quantity of the secretion (Oswald, 1958) and its gelatinous quality (Lopez-Vidriero *et al.*, 1973) are important features of the restrictive airways diseases. At the molecular level, the acidic and neutral glycoproteins are recognized as being responsible for the mucous properties either directly (Gibbons & Glover, 1959; Sturgess *et al.*, 1971) or by virtue of their association with proteins to form gels (Gibbons, 1969).

A major obstacle in studying secretory glycoproteins is the difficulty of obtaining them free from the proteins with which they are associated, often intimately, in the gelatinous secretion; most non-destructive methods give incomplete separations.

Density-gradient-equilibrium methods were successfully used for separating and characterizing the glycoprotein components of many ovarian-cyst fluids (Creeth & Denborough, 1970; Denborough & Creeth, 1970; Bhaskar & Creeth, 1974), which are secretions possessing many similarities to sputum. Moreover, the caesium salts used in the procedure tend to dissociate non-covalent protein-glycoprotein complexes (Starkey *et al.*, 1974). The method can be used on a small scale so that individual samples rather than pooled material can be examined. Accordingly we report here a study of sputa from asthmatic and chronic bronchitic patients based on density-gradient methods of separation and characterization.

Experimental

Materials

Reagents were of analytical grade, where obtainable, and were used without further purification. The CsBr (BDH, Poole, Dorset, U.K.) was of spectroscopic quality. A phosphate/chloride buffer, $I0.10$, pH 6.8 ($16.7\text{ mM-Na}_2\text{HPO}_4/16.7\text{ mM-NaH}_2\text{PO}_4/33\text{ mM-NaCl}$, containing also 1 mM-NaN_3 and, for the later samples, 1 mM-EDTA), was used as solvent for all separations and characterizations of native glycoproteins. For thiol-reduction studies a phosphate buffer, pH 8.0, $I0.10$ ($32.73\text{ mM-Na}_2\text{HPO}_4, 1.83\text{ mM-NaH}_2\text{PO}_4$) was generally used. Dithiothreitol was obtained from BDH.

Analytical density-gradient ultracentrifugation

A Beckman model E ultracentrifuge was used with schlieren optics, methods previously described being followed (Creeth & Denborough, 1970; Bhaskar & Creeth, 1974). Solutions were often run in pairs, wedge windows being used to effect image separation; however, since the zones were often hypersharp, the photographic records showed considerable overlap. In these cases, the patterns reproduced below were obtained by manual tracing of 2-fold enlargements.

CsBr was used as the gradient-forming salt, for

reasons given below; this salt has a low value of the salt-distribution parameter β (about 0.7×10^9 at 1.5 g/ml ; Ifft *et al.*, 1970) and accordingly gives fairly steep gradients when centrifuged at $40\,000\text{--}45\,000\text{ rev./min}$. Thus the span of the gradient in the analytical cell is quite high and aqueous CsBr is accordingly well suited for examining systems showing a wide spread of buoyant density, although it is not possible to band the protein and the glycoprotein in a single experiment. To band the protein, a density of approx. 1.3 g/ml is suitable, when the glycoprotein components are concentrated at the base of the cell. Correspondingly, a solution of density approx. 1.5 g/ml bands the glycoprotein, the free protein being concentrated at the meniscus. Usually, the difference in density is sufficient to ensure a small separation between the tail of the protein distribution and the less-dense region of the glycoprotein.

Sedimentation-velocity experiments were performed with the MSE Centriscan ultracentrifuge, recording with schlieren optics. Sedimentation coefficients were determined from the peak positions, by generally established procedures (e.g. Creeth, 1964), but with the use of a computer program that allowed for the finite time of scan.

Preparative experiments were performed with a Beckman model L ultracentrifuge with a type 50 titanium rotor and 13.5 ml cellulose nitrate tubes. The tubes were completely filled and centrifuged for $48\text{--}72\text{ h}$ at $42\,000\text{ rev./min}$ ($116\,000\text{ g}$, r_{av} 5.9 cm) at 8°C . Earlier experience had shown that the distribution at 48 h is only marginally different from that at 5 days , when values characteristic of equilibrium are attained (Creeth & Denborough, 1970).

Analytical methods

Amino acids were determined by Dr. A. S. R. Donald, earlier procedures being followed (Donald, 1973). The carbohydrate components were determined by g.l.c. of the trimethylsilyl derivatives, after methanolysis with 1 M-HCl for 24 h at 85°C (Clamp, 1974; I. Das, unpublished work). Solutions for analysis were prepared from samples dried to constant weight at 60°C *in vacuo* over P_2O_5 .

Reduction by thiols

Glycoprotein solutions at $0.5\text{--}1.0\text{ mg/ml}$ were treated with 0.01 M -dithiothreitol at pH 8.0 (Konigsberg, 1972). Reaction was usually complete in all conditions within 1 h , as judged by viscometric measurements. For analysis, the reduced materials were stabilized by alkylation with excess iodoacetamide, and dialysed exhaustively against water.

Viscometry

Measurements were made in the folded-capillary type of Ostwald viscometer, linked to a Hewlett-Packard autoviscometer, as described previously

(Creeth & Holt, 1972). As insufficient material was available for determining concentration-dependence, measurements were made at only a single low concentration (0.2–0.4 mg/ml) and are reported as η_{red} accordingly.

Sputum samples

With one exception, these were of mucoid (i.e. non-purulent) character (Charman & Reid, 1972). Sample 14 was classified as mucopurulent. Samples were obtained from individual patients. The diagnosis of chronic bronchitis was made in terms of the Medical Research Council Report (1965). Patients nos. 2, 7–14 and 21 were chronic bronchitics, 3, 5 and 20 extrinsic asthmatics and 4, 6, 17 and 18 intrinsic asthmatics. Patient no. 1 exhibited both chronic bronchitis and intrinsic asthma. Patient no. 19 exhibited bronchorrhea (Keal, 1971), as also did patients nos. 3–6, 17, 18 and 21. Samples were collected into sterile polyethylene containers over 1–3 h, frozen immediately and stored at -20°C for up to 6 months before use. Mucoid sputa are stable for some hours at room temperature (20°C) by rheological criteria (Sturgess, 1969).

Dispersal of sputum and preliminary examination

Although CsCl was fully effective in dispersing cyst fluids without denaturation (Creeth & Denborough, 1970; Denborough & Creeth, 1970), attempts to disperse gelatinous sputa in CsCl were unsuccessful, stringy precipitates being formed, concomitant with a large decrease in viscosity. In CsBr, however, complete dispersal could usually be effected while maintaining a high degree of viscoelasticity. The usual procedure, after thawing the frozen sample at 37°C , was to mix the sample with about twice its weight of phosphate/chloride buffer together with sufficient CsBr to give a weight fraction of 0.44, giving a final solution density of approx. 1.50 g/ml [International Critical Tables (1932) McGraw-Hill, New York]. The mixture was then gently stirred by repeated passage through a wide-bore (approx. 3 mm) Pasteur pipette; stirring was continued for periods of about 10 min at intervals for up to 24 h at 4°C , after which all samples appeared homogeneous. The solutions so obtained were usually slightly opaque, very viscous and often noticeably visco-elastic.

A sample of the CsBr solution (approx. 0.8 ml) was then removed for analytical density-gradient centrifugation. In several cases, a further sample was diluted to a weight fraction of CsBr of 0.33, giving a solution density of about 1.33 g/ml, and this was examined similarly. Determinations of the buoyant densities were made by standard methods [Creeth & Denborough (1970); note that r_0 outside the parentheses in eqn. (1) of that paper should be r_c]. Values of

β determined experimentally by Ifft *et al.* (1970) were used.

Initial fractionation

From the density-gradient pattern exhibited by the glycoprotein component, a suitable density was estimated for the separation of protein from glycoprotein in the preparative ultracentrifugation. Values were usually close to 1.40 g/ml, and the sputum solution in CsBr was diluted accordingly.

After 48 h centrifugation at 42000 rev./min, three zones were usually distinguishable by eye. The upper third was slightly yellowish but non-viscous, sometimes containing a dark band at its upper end, the middle third was usually clear and non-viscous, but sometimes contained a region of insoluble particulate material, and in one case a zone of gel; the lowermost third contained a very viscous zone. At this stage the tubes were usually sliced at the mid-point, giving fractions designated 'protein' (upper half) and 'glycoprotein' (lower half). Occasionally the tubes were divided into three, giving protein (upper half), glycoprotein 1 (third quarter) and glycoprotein 2 (fourth quarter).

After pooling corresponding fractions from different tubes, the solutions were dialysed against water until nearly free of CsBr, and then against the phosphate/chloride buffer. After removal of samples for physicochemical examination, the remaining glycoprotein fraction was either stored at -25°C or dialysed against water until salt-free and freeze-dried.

In many cases, the glycoprotein fraction from the first CsBr preparative centrifugation was subjected to further fractionation in the same manner, by using CsBr, CsCl or mixtures of CsCl and guanidinium chloride. In the latter case, the weight fraction of guanidinium chloride was 0.18, giving the dissociative conditions described by Hascall & Sajdera (1969).

Results

Analytical density-gradient ultracentrifugation of sputa

Representative patterns obtained are shown in Fig. 1. All the chronic-bronchitic sputa showed well-defined zones, often with a hypersharp region, and usually indicating two or more components of closely spaced buoyant density; in one case (sample 9) a distinct partly insoluble component of lower density was also seen. The buoyant-density values are shown in Table 1. The glycoprotein concentrations are in the range 1–3 mg/ml, but the protein is more variable (cf. the width of the unresolved zones at the meniscus).

The patterns obtained from sputa of asthmatic patients (Fig. 1b) are generally very similar to those from sputa of chronic bronchitic patients, the glycoprotein zones being well resolved and having similar buoyant densities. Samples from most of the intrinsic

Fig. 1. *Analytical density-gradient patterns of sputa after dispersal in CsBr, initial density approx. 1.50 g/ml* Number of patient is given under each frame; buoyant densities of banded components are given in Table 1. Patterns were obtained at 24–30 h at 39460 rev./min, 25°C. (a) Chronic bronchitis; (b) asthma. Curved baseline near meniscus in first pattern probably arises from leakage between compartments of cell, not observed in any other case.

Table 1. *Fractionation details and buoyant-density values in sputa and fractions*

All ρ_0 values were determined in CsBr; units are g/ml. Abbreviation: GuCl, guanidinium chloride. CsBr+CsCl $\times$ 1 (or $\times$ 2) means that the sample was obtained by first-stage separation in CsBr followed by one or two similar separations in CsCl.

Sputum no.	ρ_0 in sputum	Density-gradient separations (type and no.)	Fraction no.	ρ_0 in fraction
2	1.484	CsBr	Whole	1.491
5	1.497	CsBr	Whole	1.500
12	1.487 1.506	CsBr+CsCl $\times$ 2	Whole	1.513
13	1.487 1.502	CsBr+CsCl $\times$ 2	1 2	1.508 1.536
14	1.512 1.530	CsBr+CsCl $\times$ 2	1 2	1.514 1.536
18	1.507 1.522	CsBr+CsCl $\times$ 1 CsBr+(CsCl/GuCl)	1 2 1*	1.520 1.548 1.520
19	1.496	CsBr+CsCl $\times$ 1 CsBr+(CsCl/GuCl)	1 1*	1.500 1.503
20	1.487	CsBr+CsCl $\times$ 1 CsBr+CsCl $\times$ 1	1 2 1†	1.492 1.512 1.485
21	1.498	CsBr CsBr+CsCl $\times$ 1	Whole Whole	1.501 1.509

* Second-stage centrifugation in CsCl/GuCl.

† Gel fraction (see the text).

asthmatic patients showed hypersharp zones and two or more glycoprotein components; in one case (sample 6) a well-defined denser component is seen also. On the other hand, samples from two asthmatic patients (1 and 20) and from one case of bronchorrhea (19) showed generally lower concentrations and only a single, nearly symmetrical, glycoprotein zone.

The patterns shown in Fig. 1 were obtained after periods of approx. 30 h at the set speed (usually 39460 rev./min). Extensive experience of glycoprotein systems in general, and direct evidence from some of the fractions prepared in this work, show that this period is adequate to establish primary isopycnic equilibrium; i.e. the caesium salt and all free macro-

molecular species attain time-independent distributions, within experimental error. With some sputa, considerable changes in the density-gradient distribution occurred after the 30h period; these are described below. For the present purposes, the patterns shown in Fig. 1 may be regarded as illustrating the composition existing in the original sputum.

Of the 19 sputa examined, nine were selected for fractionation, these being fully representative clinically and in physicochemical terms.

Separation of the protein and glycoprotein components

When CsCl was used for separating cyst-fluid glycoproteins by density-gradient centrifugation, no free protein could be detected in the analytical isopycnic patterns of the glycoprotein components, a test of proved high sensitivity (Bhaskar & Creeth, 1974). Although CsBr has a lower resolving power than CsCl, simulation of typical separation con-

ditions showed that it should still be quite adequate in the conditions chosen (Creeth & Horton, 1977). In an experimental test of CsBr on a cyst fluid, quantitative separation of the protein and glycoprotein components was obtained, in agreement with the theoretical prediction.

When the sputum samples were subjected to density-gradient separation in CsBr as described above, some gave clear-cut separations, but others apparently did not. Fig. 2 presents an example of successful single-stage separation; comparison of the sputum pattern at density 1.5 g/ml with that of the isolated glycoprotein shows that the steep downward curvature near the meniscus, corresponding to free protein, has disappeared in the glycoprotein fraction. Apart from a trace of denser material visible at the base of the cell, the glycoprotein fraction consists of a single sharp zone. After suspension in CsCl and a second density-gradient fractionation, the glyco-

Fig. 2. Fractionation of sputum sample 21 by single-stage density-gradient centrifugation in CsBr (see the text)

Analytical density-gradient patterns, also in CsBr, of: (a) sputum; (b) glycoprotein fraction from single-stage separation in CsBr; (c) glycoprotein fraction from (b) after second-stage separation in CsCl; (d) protein fraction from first-stage separation in CsBr. The dark band visible near the meniscus in the first glycoprotein fraction originates from a different cell, examined simultaneously.

Fig. 3. Effect of repeated density-gradient separations on glycoprotein fractions

All patterns were obtained in CsBr; upper row, sputum sample 20; lower row, sputum sample 13. (a) Glycoprotein-1 and -2 fractions (indicated by 1 and 2) from first-stage separation in CsBr; (b) as (a), after second-stage separation in CsCl; (c) gel fraction (see the text) from first-stage separation in CsBr; (d) as (c), after second-stage separation in CsCl; (e) glycoprotein fraction from first-stage separation in CsBr; (f) and (g) glycoprotein fractions 1 and 2 respectively, after two further separations in CsCl.

protein pattern is unchanged, and no liberated protein or peptide is visible at the meniscus. The protein fraction from the CsBr centrifugation is distinctly paucidisperse when examined at 1.3 g/ml, but it is almost devoid of the denser material. Thus the analytical experiments on the sputum and fractions show that there are essentially only two density classes of material in this sample, and that these are satisfactorily separated in the preparative centrifugation, which approaches its theoretical efficiency. A similarly clean separation of the glycoprotein was obtained with sputum samples 2 and 5.

Several samples gave glycoprotein components by this procedure that showed small proportions of material at the meniscus in analytical control experiments (Fig. 3). These experiments do not distinguish between free protein (buoyant density, ρ_0 , 1.3 g/ml) and low-density glycoprotein, as any component of ρ_0 lower than the value at the meniscus (ρ_a) (approx. 1.41 g/ml) will be concentrated in that region. Although a distinction is possible by modifying the conditions (higher speed or lower initial density), it was decided to remove these low-density components as far as possible, so that attention would be focused on the major glycoprotein, which usually banded within a clearly defined envelope of mean density approx. 1.5 g/ml. Accordingly, one or more additional separation experiments were performed on all first-stage preparations showing such curvature at the meniscus. After the initial dispersal in CsBr and the removal of most of the free protein, the glycoprotein fraction was readily dispersed in CsCl, and accordingly this reagent was used for subsequent centrifugations.

As noted above, CsCl has an even higher resolving power than CsBr, and accordingly any trace of residual protein in the first-stage fractions should be very easily removed. In these experiments, the initial density was approx. 1.47 g/ml, and the central 50% of the tube was usually taken as the glycoprotein fraction; in some cases, cuts were made at the second and third quarters, yielding glycoprotein-1 and -2 fractions. The products obtained thus were satisfactory by standards established with cyst-fluid glycoproteins. Nevertheless, close examination of the patterns reveals a trace of residual curvature in some cases (e.g. sample 20), even in those that had been subjected to three serial fractionations. The curvature is due to traces of low-density glycoprotein, amounting to less than 0.5% of the main banded component. It is present because the centrifugation in CsCl yields broader glycoprotein zones than in CsBr, although CsCl segregates proteins more sharply than CsBr (Creeth & Horton, 1977).

An insoluble gel component was isolated from the middle of the tubes in the CsBr preparative centrifugation with sample 20, at about ρ 1.4 g/ml; on freezing and thawing this spontaneously dissolved and

its analytical pattern is shown in Fig. 3, together with the pattern after a subsequent centrifugation in CsCl. The final product is also seen to give a single symmetrical zone.

Sample 14, the only mucopurulent sample subjected to separation, contained an appreciable amount of the very dense component, traces of which were noted in several other samples. After separation from the glycoprotein by a density-gradient centrifugation at ρ 1.5 g/ml, it formed an unsymmetrical moderately sharp zone at ρ_0 1.62 and was identified as DNA by the diphenylamine colour reaction (Croft & Lubran, 1965) and its absorption maximum at 260 nm. In CsBr, DNA usually bands at about 1.63 g/ml (Ifft, 1973); from its appearance the DNA was extensively degraded. The final glycoprotein-1 fraction from sample 14 was devoid of both protein and nucleic acid.

In two cases, a comparison was made of the effect of CsCl alone with that of a mixture of CsCl with guanidinium chloride (Hascall & Sajdera, 1969): one of these was a sputum where the original analytical experiment in CsBr had shown the time-dependent effects discussed below, whereas the other had been normal in this respect. The results, including the final (48 h) patterns for the sputa, are shown in Fig. 4. The glycoprotein-1 fractions produced by the mixed electrolyte are evidently very similar to those produced by CsCl alone, the only discernible difference being a slight diminution, in the product of the mixed electrolyte, in part of the positive lobe; this would correspond to the loss of a small proportion of one of the less-dense components.

The fractionation treatments of the various sputa are summarized in Table 1, which also lists essential buoyant-density data. Except for samples 12 and 21, the glycoprotein fraction was subdivided after the CsBr stage, the two subfractions subsequently being treated in parallel. The glycoprotein-2 fractions, which constituted less than 25% of the total glycoprotein, usually contained a substantial proportion of the glycoprotein-1 component in addition to the denser component specified in Table 1. The glycoprotein-1 components finally obtained, whose buoyant densities are listed in the last column of Table 1, are thus the main products of the separation; their relationship to the glycoprotein components visible in the sputa is made clear by inspection of the original patterns (Fig. 1) and by comparison of the buoyant densities before and after separation. In some cases (e.g. sample 21), the density-gradient patterns of sputum and glycoprotein-1 fraction are obviously similar, and in these cases (i.e. also samples 2 and 5) the buoyant density of the fraction is either identical with, or only slightly greater than, that of the main glycoprotein component of the sputum. It is significant that these samples were found to separate cleanly by a single-stage centrifugation in CsBr.

Fig. 4. Use of CsCl/guanidinium chloride mixture as a second-stage density-gradient separation medium

All patterns were obtained in CsBr; upper row, sample 18; lower row, sample 19. (a) and (d) Density-gradient patterns of original sputa; in (a), part of an unrelated pattern from a different cell is visible. (b) and (e) Glycoprotein-1 fractions from first-stage separation in CsBr. (c) Lower and (f) upper, as (b) and (e) respectively, after second-stage separation in CsCl; (e) upper and (f) lower, as (b) and (e) respectively, after second-stage separation in CsCl/guanidinium chloride mixture.

In other cases (e.g. samples 12, 13 and 18), the relationship is less clear, and the buoyant density of the isolated glycoproteins is noticeably greater than the lower of the two values in the sputum; this observation is associated with the time-dependent effects referred to previously. The range of these effects observed with the sputa is shown in Fig. 5, referring to samples 18 and 14. Sample 12 behaved similarly, and a less-marked effect was observed with sample 13, the spike evident in Fig. 1 disappearing at 48h. All these sputa behave similarly: a pattern at 24–30h, in which two glycoprotein components are clearly resolved, is transformed in 50–90h into one showing only a single component, this being the denser of the two initially visible; often hypersharp spikes on the pattern disappear. As the less-dense component decreases in concentration, so the denser component increases. Clearly, the slow removal of protein or peptide from a complex between a protein and a denser glycoprotein could account qualitatively for the transformation, and such behaviour would be in keeping with the known characteristic of this type of secretion (Snary & Allen, 1971; Starkey *et al.*, 1974).

Further physicochemical characterization

The apparent molecular weight determined from the density-gradient patterns, M_w^{app} , is not directly

related to the molecular weight of a material that is disperse in density; nevertheless, together with the weight-average molecular weight for the whole distribution, M_w^0 , it provides a useful qualitative index of dispersity in density. Accordingly, both M_w^{app} and M_w^0 were determined for the fractions whose homogeneity was judged adequate. Sedimentation-velocity experiments were performed on all fractions; rapidly spreading peaks were observed at the low concentration used, sometimes with an indication of discrete components of higher sedimentation coefficient than the main component. Representative patterns are shown in Fig. 6. The results of the physicochemical measurements are summarized in Table 2: the $s_{25,w}$ and M_w^0 values show that these glycoproteins are of very high molecular weight.

Analytical characterization of the glycoprotein fractions

The final isopycnic patterns for the glycoprotein or glycoprotein-1 fractions from samples 2, 5, 12, 13, 14, 17, 20 and 21 showed that these materials are essentially unimodal in density distribution, and, although the sedimentation-velocity patterns indicated considerable polydispersity in size, it is the former property that is sensitive to analytical variation (Creeth *et al.*, 1974). Thus the analyses for these fractions are directly interpretable. The glycoprotein-2 fractions in general, and both glycoprotein-1 and -2 fractions for sample 19, are more disperse in density,

Fig. 5. Slow variations in density-gradient patterns of sputa

Upper row, sample 18; lower row, sample 14. Sample 18 shows the elimination of a very sharp spike at around 24h, followed by the disappearance of the less-dense glycoprotein component at 48h. Sample 14 at 24h shows predominantly less-dense glycoprotein, changing at 50h to approximately equal proportions; at 90h only the more dense is visible. Patterns are in CsBr, initial density 1.50g/ml, 39460 rev./min, 25°C.

Fig. 6. Representative sedimentation-velocity patterns obtained with glycoprotein fractions (a) Sample 13, glycoprotein 1; (b) sample 20, glycoprotein 1; (c) sample 14, glycoprotein 1; (d) as (c) after reduction with dithiothreitol. All patterns were obtained with solutions of approx. 1 mg/ml at 25°C, in 20mm cells of the Centriscan ultracentrifuge at 35000 rev./min; scans were recorded at 4 min intervals.

Table 2. Physicochemical characterization of the major bronchial glycoprotein fractions, and effect of thiol reduction on representative fractions

All values in parentheses refer to reduced materials. n.d., Not determined. $\eta_{red.}$ was determined at pH 8.0, except for values denoted †, which were at pH 6.8 and reduction was by mercaptoethanol. $M_{d.g.}^{app.}$; +, (—): value of apparent molecular weight derived from positive (negative) lobe of density-gradient pattern.

Glycoprotein fraction	...	12	13 (1)	14 (1)	17 (1)	18 (1)	18 (1*)	20 (1)	20 (†)	21
ρ_0 (g/ml)		1.513 (1.519)	1.508 (1.510)	1.514 (1.518)	1.496	1.520 n.d.	1.520	1.492 (1.512)	1.485	1.509
$10^{-3} \times M_{d.g.}^{app.}$	+	405 (322)	618 (652)	207 (244)	396	133	117	322 (133)	169	546
	—	164 (147)	394 (252)	520 (192)	196	203 —	187 —	176 (190)	154	348
$s_{25,w}$ (S)		18.0 (9.5)	23.3 —	19.7 (10.0)	19.9 —	17.6	18.8	17.5 (10.8)	13.6	—
$10^{-6} \times M_w^0$		5.1 (1.3)	7.0 (2.3)	5.8 (0.57)	3.3 (1.1)	—	—	5.1 (0.78)	—	—
$\eta_{red.}$ (ml/g)		390 (61)	268 (80)	507; 252† (106); (48)†	244 (60)	—	—	340 (84)	—	—

*, † as in Table 1.

Table 3. *Analytical composition of the bronchial glycoproteins*
Results are expressed as g of anhydro component/100 g of glycoprotein. ^{a-d} Totals include sulphate contents of 8, 6, 8 and 5% respectively.

Sputum no. ... Glycoprotein fraction ...	12	13	14	17	18	19	20	21
	Whole	1	2	1	1*	1	1†	Whole
Fuc	8	8	12	14	7	11	7	10
Gal	21	21	9	16	17	20	3	10
GlcNAc	22	20	22	26	18	23	20	23
GalNAc	9	23	9	22	18	19	18	23
Glu	—	8	11	14	9	6	14	6
Man	—	0.4	0.2	0.9	1.2	1.2	0.5	0.8
AcNeu	0.6	0.7	0.8	1.2	1.0	1.8	0.2	1.0
Amino acids	23	12	0.1	1.3	1.4	2.4	1.5	1.3
Total	103	97	96	99	88 ^a	88	102	86 ^d
			98	101	93 ^b	93	85	
			97	99	88 ^a	88	109	
			98	101	93 ^b	93	109	
			97	99	88 ^a	88	109	

^a, [†] as in Table 1.

and accordingly the analytical values will represent averages over the components resolved in the patterns.

The results of the analyses, expressed in terms of percentage composition of peptide and the five sugar components (fucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid), making allowance for water lost on peptide or glycosidic-bond formation, are given in Table 3, and the detailed amino acid distribution of the peptide component is given in Table 4. Results where additivity was unsatisfactory, or where the component was a minor fraction, have been omitted.

In addition to the components specified above, small amounts of glucose and mannose were detected in the chromatograms; their total did not exceed 2% in the main fractions, but the values are included in Table 3 because their consistent occurrence suggests that these sugars are indeed minor components of the glycoproteins.

Significant proportions of sulphate were found in some fractions containing little sialic acid; however, insufficient material was available for the sulphate analysis (Antonopoulos, 1962) to be extended to other fractions.

The most significant features of the analytical composition are as follows.

(i) The glycoprotein-1 fractions contain a relatively high proportion of peptide, ranging between 22 and 30%. This is considerably higher than the average of 15% characterizing, for example, the purified soluble blood-group-specific glycoproteins of human ovarian-cyst fluids (Watkins, 1972; Donald, 1973), but similar to the values for the sparingly soluble or insoluble cyst-fluid glycoproteins, for which values of 30% peptide are common (Dunstone & Morgan, 1965; Dunstone, 1969).

(ii) The glycoprotein-2 fractions have a significantly lower proportion of peptide.

(iii) The amino acid profiles of all fractions are remarkably similar, being characterized particularly by values for the sum of (Ser+Thr+Pro) of around 40%. Differences between the various preparations are seen most clearly in the values for the acidic amino acids, where (Asp+Glu) varies from 12 to 21%. Table 3 also shows that the (Asp+Glu) sum is highest for those fractions of lowest (Ser+Thr+Pro), an observation in keeping with the well-known correlation between amino acid profile and total peptide content. Similarly, the cystine content of all fractions is appreciable; cystine is often absent from readily soluble glycoproteins, but is found in those that are sparingly soluble (Dunstone & Morgan, 1965; Donald, 1973; Masson & Heremans, 1973).

(iv) The content of fucose is variable, those of galactose and *N*-acetylglucosamine are much the same as in other mucus glycoproteins (e.g. Donald, 1973; Starkey *et al.*, 1974), whereas the sialic acid content is

Table 4. *Amino acid composition of bronchial glycoproteins*
Results are expressed as mol of amino acid/100 mol of total amino acids. Values in parentheses refer to thiol-reduced materials.

Sputum no.	12	13		14		17		18		19		20		21
Glycoprotein fraction	Whole	1	2	1	2	1	2	1	1*	1	1*	1	1†	Whole
Asp	7.3	6.8	6.5	6.3	7.2	7.3	5.7	8.6	8.3	10.8	10.1	9.2	6.5	7.3
Thr	17.6	17.4	18.7	19.5	18.1	20.7	22.5	17.9	18.1	13.3	13.0	17.1	21.5	15.0
Ser	12.1	10.6	11.2	11.6	10.1	12.0	11.7	12.3	12.7	10.9	11.8	13.4	15.4	10.5
Glu	7.3	8.2	7.7	7.2	9.9	8.1	6.7	8.2	8.1	10.3	10.9	9.4	7.6	9.7
Pro	13.0	12.5	12.5	13.8	15.9	10.0	11.0	10.2	10.5	11.9	13.8	11.4	11.1	11.1
Gly	7.9	7.7	7.1	5.9	4.9	7.5	7.3	9.5	7.6	9.5	10.6	7.3	7.0	9.2
Ala	8.2	7.4	7.7	6.2	7.7	7.6	8.1	6.9	7.2	6.8	3.6	5.9	7.1	6.7
Cys	1.1	2.3	2.8	2.7	1.2	2.2	3.3	2.4	2.6	1.6	1.8	2.8	0.2	1.7
				(0.9)								(1.0)		
Val	5.0	4.5	4.4	3.5	3.1	5.1	5.4	4.9	4.8	5.0	4.5	5.1	5.0	4.3
Met	0.1	0.8	0.8	0.8	0.9	0.03	0.1	0.4	0.4	0.2	0.3	0.02	0.6	0.3
Ile	3.0	2.4	2.3	2.8	2.5	2.1	2.1	2.1	2.1	1.7	1.9	2.2	2.6	1.7
Leu	5.8	6.0	6.0	5.4	4.9	5.0	4.9	5.2	4.9	5.3	5.2	3.9	3.0	4.8
Tyr	1.7	1.6	1.6	1.7	1.6	1.6	1.4	1.6	1.8	2.0	1.7	1.7	1.6	1.4
Phe	2.3	2.3	2.3	2.0	2.0	2.6	2.0	2.3	2.1	2.5	2.1	2.4	2.0	2.3
His	1.2	3.3	2.8	3.6	4.0	3.0	3.3	1.8	2.5	2.3	2.2	1.8	1.9	3.2
Lys	2.1	2.3	1.8	2.9	2.4	1.6	1.6	2.1	2.1	2.0	2.2	2.0	2.7	2.2
Arg	4.3	3.8	3.7	4.0	3.9	3.6	3.1	3.9	4.2	3.9	4.2	4.2	4.1	3.3
Ser+Thr+Pro	42.7	40.5	42.4	44.9	44.1	42.7	45.2	40.4	41.3	36.1	38.6	41.9	48.0	40.3
				(53.1)								(52.3)		
Asp+Glu	14.6	15.0	14.2	13.5	17.1	15.4	12.4	16.8	16.4	21.1	21.0	18.6	14.1	18.4
				(10.4)								(14.2)		

*, † as in Table 1.

extremely variable, reaching quite high values. Relatively little difference occurs between the glycoprotein-1 and -2 components of a sputum sample in terms of carbohydrate composition.

(v) The products obtained from a second-stage centrifugation in guanidinium chloride/CsCl mixture are analytically and physically indistinguishable from those obtained with CsCl alone. Since the mixed solvent is more strongly dissociative, yet removes no more peptide, it must be concluded that the CsCl products are also devoid of non-covalently bound protein, in agreement with the analytical density-gradient experiments on these materials.

Reduction by thiols

Representative glycoprotein fractions were reduced by dithiothreitol, as described above. The decrease in viscosity was immediately apparent, and usually was too rapid to be followed experimentally; in one case, however (sample 20, glycoprotein 1), several hours were required to reach a constant value. Measurements were made of the reduced viscosity, sedimentation coefficient at a low concentration, weight-average molecular weight and buoyant density. The results are included in Table 2. The values of η_{red} in Table 2 are valid only for the comparison of native and thiol-reduced materials; minor differences in concentration prohibit the direct comparison of different fractions. The values in Table 2 for the native and reduced forms of any fraction illustrate clearly the marked decrease in size brought about by thiol reduction; if faster components were evident in the sedimentation-velocity patterns of the native materials (e.g. sample 14, glycoprotein 1), these disappeared on reduction (Fig. 6).

The buoyant densities are consistently increased by a small amount on reduction, and, correspondingly, the density-gradient patterns show a slight increase in free protein. The patterns in Fig. 7 illustrate the various effects observed: (i) the increased buoyant density of the reduced material, (ii) the increase in area of the substance concentrated at the meniscus, and (iii) the very sharp zone, which includes precipitated material, given by the native glycoprotein at this pH; the buoyant density, however, was identical with that observed at pH 6.8. At the lower pH (Fig. 3), the pattern was much less sharp, and indeed the M^{app} values given in Table 2 confirm that the spreading of the reduced material is little more than that of the native at pH 6.8, although the molecular weight is greatly decreased. The simulation shown in the upper part of Fig. 7 shows that the single value of 180000 for M^{app} gives a good fit to the observed curve for the main component, and inclusion of 6% of a protein component (ρ_0 1.31) of M^{app} 100000 reproduces the distribution at the meniscus within experimental error. The smaller area in the

Fig. 7. Thiol reduction of a bronchial glycoprotein: analytical density-gradient patterns of native and reduced materials in CsBr, both at pH 8.0

Results were obtained with glycoprotein 1 from sputum sample 20: (a) native; (b) reduced (see the text); (c) simulation of pattern of reduced glycoprotein; the points were calculated on the basis of a glycoprotein component of mol.wt. 180000, ρ_0 1.506 g/ml, initial concn. 0.85 mg/ml (fully banded pattern) and a protein component of mol.wt. 100000, ρ_0 1.31 g/ml, initial concn. 0.05 mg/ml, in a medium of initial density 1.483 g/ml at 39460 rev./min and 25°C, as used in (b).

corresponding region of the cell containing native material can be simulated by a trace amount (less than 0.5%) of a similar protein. It is provisionally concluded, therefore, that protein of mol.wt. approx. 10^5 is liberated at least to the extent of 5% of the glycoprotein mass by thiol reduction, with a simultaneous decrease in molecular weight by a factor, in this case, of at least 6. The amino acid profiles for two of the thiol-reduced materials show a significant loss of acidic amino acids and cystine with concomitant gain in relative proportion of serine, threonine and proline (results included in Table 4). No other amino acid changed significantly. The liberated protein is therefore relatively richer in (Asp+Glu) and poorer in (Ser+Thr+Pro) than the native glycoprotein. S-Carboxymethylcysteine was not detected in the analysis of the reduced alkylated materials, although trials with an authentic sample confirmed its stability and detectability at the expected concentration.

Stability

The purified glycoprotein preparations were freeze-dried after exhaustive dialysis against water, and subsequently stored at room temperature. Such preparations were stable for several months, but on storage for about 1 year some were found to contain a high proportion of material insoluble in water or caesium salts. Storage *in vacuo* and at low temperature is therefore to be recommended.

Discussion

The results described above show that the density-gradient method is successful as a means of separating and purifying the glycoprotein component of sputa, although not as readily as with ovarian cysts. The slow and incomplete separation often observed in single-stage density-gradient separation in CsBr is probably a consequence of a very close association between the protein and glycoprotein components in the secretion, as postulated by current theories of the rheological properties of sputum (Charman *et al.*, 1974). Because the two components are eventually separable in a density gradient, it follows that the association is non-covalent, and electrostatic forces, hydrogen bonds and hydrophobic bonds are therefore implicated. Separation by a density gradient is expected to be more efficient than by gel chromatography because the kinetic forces operative in both processes are supplemented by the buoyant constraints of the gradient; moreover, the high ionic strength and partially chaotropic nature of CsBr (Hatefi & Hanstein, 1969; Noelken, 1970) are expected to lessen electrostatic attraction and destabilize the other types of bond referred to (Tanford, 1968; Sawyer & Puckridge, 1973).

Multi-stage density-gradient centrifugation, however, is expensive and tedious, and CsBr, although satisfactory as a dispersant, cannot therefore be regarded as an ideal reagent. LiBr, used successfully for dispersing and fractionating proteoglycans and cervical mucins (Robinson & Hopwood, 1973; Gibbons & Sellwood, 1973), has theoretical attractions in spite of the difficulties arising from the low gradient (Creeth & Horton, 1977); we found, however, that it caused precipitation of some glycoprotein fractions. The strongly dissociative mixture of CsCl/guanidinium chloride might be more effective, and, as the results show, is apparently without action on the isolated glycoproteins. However, the possibility of incorporating denatured protein into a covalent complex with the glycoprotein by thiol-disulphide exchange is a formidable objection to its use with sputum itself.

In comparison with other preparations of bronchial glycoproteins [e.g. those of Boat *et al.* (1976), Roberts (1974), Lamblin *et al.* (1973) and Degand *et al.* (1973)], our materials are of higher molecular weight

and generally greater peptide content; moreover, the amino acid profile resembles those of the less-soluble cyst glycoproteins. In this respect, they are similar to the most gelatinous of Roberts's (1974) fractions. From the nature of sputum, the possibility of enzymic degradation cannot be eliminated; however, the addition of large amounts of CsBr (together with some NaN₃) immediately on thawing the samples, and subsequent processing at as low a temperature as possible, should minimize enzymic attack in the period before the protein is separated. With this reservation, our materials should be closer to a native condition than those obtained by degradative processes such as thiol treatment, sonication or deliberate proteolytic digestion. Although Snary *et al.* (1974) have suggested an irreversible effect of caesium salts on glycoproteins, the evidence rested on a comparison of the properties of a mucoprotein before and after exposure to CsCl, during which treatment free protein was lost. Ovarian-cyst glycoproteins were apparently unchanged by exposure to CsCl or CsCl/guanidinium chloride (Bhaskar & Creeth, 1974). It is therefore believed that the products now obtained, which contain no more than a trace of free protein, are in a native condition as regards primary structure. Since the non-covalently bound protein fulfils a structural role in the mucus gel, its removal probably causes significant changes in the spatial arrangement of the glycoprotein, so in this sense no protein-free product can be of native configuration.

The thiol-reduction results indicate that these glycoproteins are disulphide-linked aggregates. This confirms the more detailed findings of Roberts (1976), and shows the general similarity of bronchial glycoproteins to those of gastric mucin (Snary *et al.*, 1974) and cervical mucin (Hatcher *et al.*, 1977). As all species, both before and after thiol reduction, are polydisperse, whole-number ratios between the molecular weights are not to be expected. The values given, however, suggest that three to ten of the smaller species are linked in the parent glycoprotein. The changes in buoyant density (Table 2) and analytical composition (Table 4) indicate a small but significant loss of peptide on thiol reduction, and thereby suggest that the disulphide bridging occurs at least in part through a cross-linking protein; Roberts (1976) showed that immunoglobulin A and other serum proteins were involved in such links. The liberation of peptide by thiols provides a straightforward interpretation of the differences in amino acid profile between native materials and those obtained by thiol reduction. Since Roberts (1974) was unable to separate his bronchial glycoproteins into neutral and acidic fractions by ion-exchange chromatography, whereas Havez *et al.* (1967) were able to resolve the thiol-reduced glycoproteins by electrophoresis, it is possible that the neutral and

sialo-mucins are produced at separate sites and later aggregated by oxidation of cysteine thiol groups. This would be in agreement with the suggestion of Boat & Matthews (1973) on the functional significance of the gelatinous component of the bronchial mucins, that is to protect the inner regions of the mucous blanket from oxidation and maintain them in soluble reduced form.

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