Biochem. J. (1983) **211**, 13–22
Printed in Great Britain

Isolation and characterization of human cervical-mucus glycoproteins

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(*Received 6 July 1982/Accepted 23 November 1982*)

Mucus glycoproteins (mucins) were extracted from human cervical pregnancy mucus by 6 M-guanidinium chloride in the presence of proteinase inhibitors. Purification was subsequently achieved by isopycnic density-gradient centrifugation in CsCl/guanidinium chloride gradients. The purified macromolecules represented approx. 85% of the total and were devoid of nucleic acids and proteins, as judged by analytical density-gradient centrifugation, disc electrophoresis and u.v. spectroscopy. Sedimentation-velocity centrifugation revealed a single unimodal peak with $s_{20,w}$ 50.1 S in 0.2 M-NaCl and 37.0 S in 6 M-guanidinium chloride. Molecular weights obtained by light-scattering were 9.7×10^6 and 5.9×10^6 in 0.2 M-NaCl and 6 M-guanidinium chloride respectively. The chemical analyses were typical of those of epithelial mucins. The macromolecules contained approx. 20% (w/w) of protein, and 65% (w/w) was accounted for as carbohydrate. Serine and threonine constituted 32 mol/100 mol and proline 10 mol/100 mol of the amino acids. The major sugars found were *N*-acetylglucosamine (12.8%), *N*-acetylgalactosamine (9.7%), galactose (18.7%), sialic acid (15.0%) and fucose (7.5%).

Cervical mucus plays a vital role in the protection of the uterine cavity and controls spermatozoal survival and migration. The amount and physical properties of mucus vary during the ovulatory cycle. At ovulation, oestrogens induce an increased hydration of mucus, which results in a watery secretion with high spermatozoal penetrability and low viscoelasticity (Wolf *et al.*, 1978). In contrast, during the luteal phase the mucus is scanty, contains less water and provides an effective barrier to the spermatozoa. During pregnancy a large mucus plug occludes the cervical canal. This mucus is related to the luteal-phase mucus rather than to that found at mid-cycle (Chretien *et al.*, 1979). For reviews see, for example, Chantler (1982) and Elstein (1982).

The mucus glycoproteins (the mucins) provide the structural framework of the mucus gel, the properties of which depend on the structural integrity of these macromolecules. Thus an understanding of how the mucins contribute to the physical properties of mucus requires that structurally intact glycoproteins are studied. Consequently, potentially destructive techniques for the solubilization of the mucus gel, e.g. proteolytic digestion, reduction of disulphide bonds, high shear and sonication, must be avoided or used with discretion. Furthermore, the likely presence of degradative enzymes in the mucus gel must be considered.

To minimize the risk of degradation during preparation, we have extracted mucus by gentle stirring in 6 M-guanidinium chloride and in the presence of proteinase inhibitors. Purification was subsequently achieved by using isopycnic density-gradient centrifugation in CsCl/guanidinium chloride. A preliminary report of this work has appeared (Carlstedt *et al.*, 1982).

Experimental

Materials

Guanidinium chloride was obtained from Bethesda Research Laboratories (ultrapure grade) and from Sigma Chemical Co. (practical grade). Stock solutions of the practical grade were treated with charcoal before use. Papain (EC 3.4.22.2; twice crystallized), ribonuclease A (EC 3.1.27.5; type IA), deoxyribonuclease I (EC 3.1.21.1; type DN-CL) and DNA (type I, calf thymus) were bought from Sigma, and di-isopropyl phosphorofluoridate was from Fluka. Polyacrylamide (enzyme grade) was purchased from Eastman Kodak, and *NN'*-methylene-

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bisacrylamide was from BDH Chemicals. Sepharose CL-4B and CL-2B were obtained from Pharmacia, and filters (nominal cut-offs 0.22 μm, 0.45 μm and 1.2 μm) were from Millipore.

Analytical methods

Hexose was determined with the anthrone method (Goa, 1955) and sialic acid as described by Jourdian *et al.* (1971). An automated version of the latter was used for fractions from column chromatography and from preparative density-gradient centrifugation (Lohmander *et al.*, 1980). Neutral sugars were determined as the corresponding alditol acetates, with L-arabinose as internal standard (Lindahl, 1970). Samples were hydrolysed in 2 M-trifluoroacetic acid at 100°C for 4 h in sealed tubes under N_2. Amino acids and hexosamines were determined quantitatively with a Durrum automatic amino acid analyser, with L-norleucine and D-glucosaminitol as internal standards respectively. Amino acids were released by hydrolysis for 24 h in 6.0 M-HCl (AristaR) at 110°C, and hexosamines for 10 h in 4.0 M-HCl (AristaR) at 100°C, in sealed tubes under argon. Cysteine was measured after performic acid oxidation (Hirs, 1967). Densities of fractions from preparative density-gradient centrifugation were assessed with a Carlsberg pipette as a pycnometer.

Polyacrylamide-gel electrophoresis was conducted on gels (total monomer concentration 10.5 g/100 ml) in 0.1% (w/v) sodium dodecyl sulphate as described by Neville (1971). The gels were stained with 0.25% Kenacid dissolved in acetic acid/methanol/water (7:25:68, by vol.).

The water content of freeze-dried mucins was determined as the loss in weight after drying for 18 h at reduced pressure over P_2O_5 at 110.6°C (refluxing toluene). Drying for longer times gave no further loss of weight.

Analytical ultracentrifugation

Sedimentation-velocity and analytical isopycnic density-gradient centrifugations were performed with an MSE 75 Centriscan analytical ultracentrifuge. Isopycnic centrifugation used to monitor the purification of the mucins was performed as described in the legend to Fig. 3. Sedimentation-velocity centrifugation of purified mucins was performed at 31000 rev./min at 20°C, with the schlieren optics. Samples were dialysed against 6 M-guanidinium chloride (ultrapure)/1 mM-Na_2EDTA/1 mM-sodium phosphate buffer, pH 6.5, or 0.2 M-NaCl/1 mM-Na_2EDTA/1 mM-sodium phosphate buffer, pH 6.5. The apparent sedimentation coefficients were determined from the peak positions, and $1/s$ was extrapolated to zero concentration (linear relationship) and corrected to standard conditions. A partial specific volume of 0.62 ml/g was used (Bhaskar & Creeth, 1974).

Light-scattering

All buffers were filtered through a 0.22 μm filter before use. Mucin solutions were dialysed at room temperature to reach constant chemical potential of diffusible components (Casassa & Eisenberg, 1964). Experiments showed that dialysed stock solutions could be diluted with the cognate diffusate without affecting the results (see also Eisenberg, 1971). After dilution, the mucin solutions and the blanks were clarified by centrifugation at 20000 g for 1–2 h. The ratio of the intensities of light scattered at 30° and at 150° was in the range of 1.05–1.10 for the blank solutions, indicating the absence of large particles.

The intensity of light scattered at angles between 30° and 150° was determined with a Sofica model 42000 photo-goniodiffusometer at 436 nm. The polymer solid standard was calibrated with redistilled benzene (AnalaR) with a Rayleigh ratio of 45.6×10^{-6} cm^{-1} and was then used to calibrate the instrument. Readings were evaluated with the corrections outlined by Tomimatsu *et al.* (1968) and plotted in accordance with Zimm (1948). Linear least-squares analyses were used to extrapolate to $c = 0$ and to $\theta = 0$, the latter values being obtained from the readings at 30°, 37.5°, 45° and 60°. Radii of gyration were calculated from the slope of the extrapolated line at $c = 0$, by using the angles above.

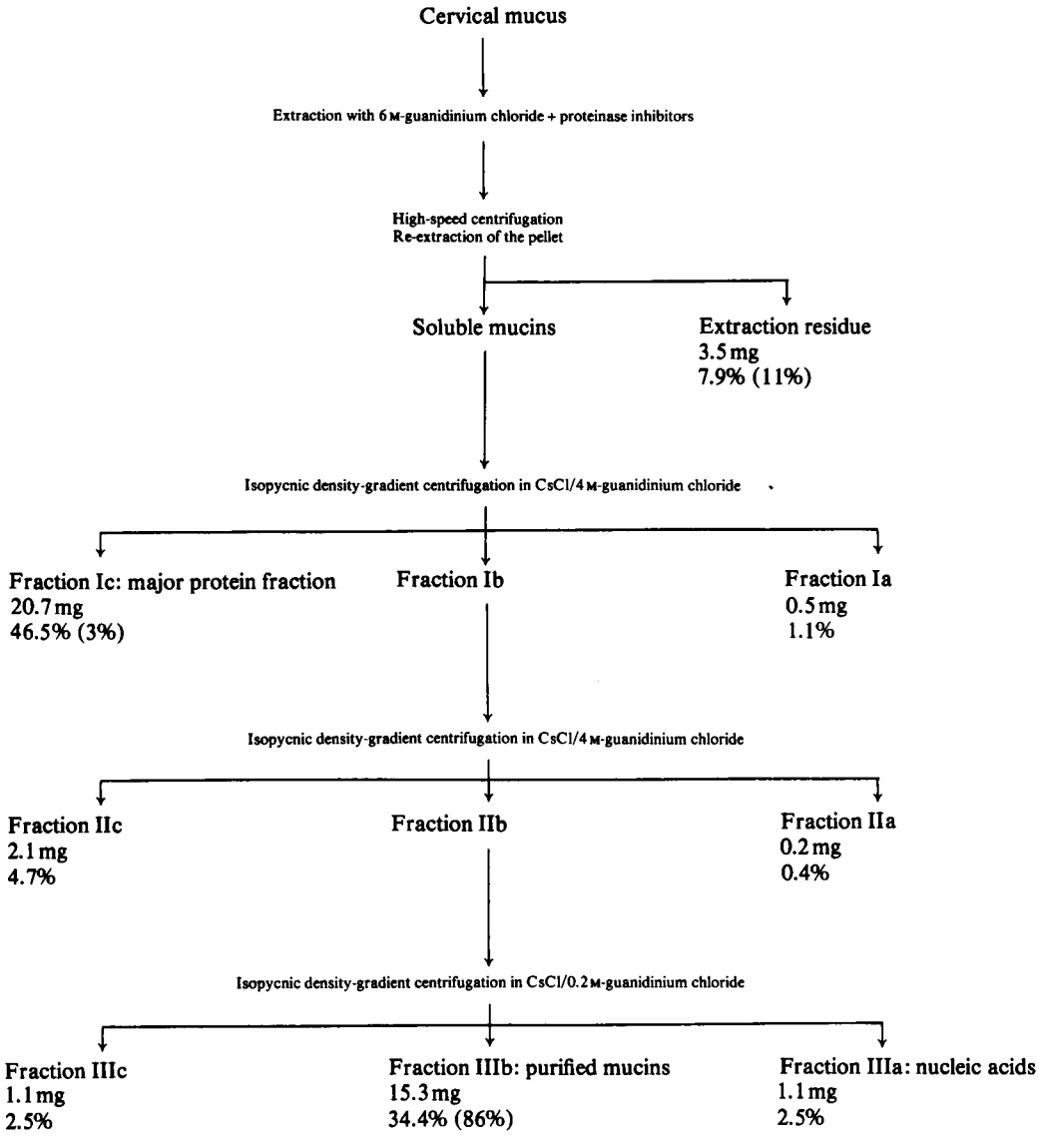
The apparent refractive-index increment at constant chemical potential of diffusible components was determined at 436 nm and 20°C with a Shimadzu model DR-4 differential refractometer. Solutions (approx. 3 mg/ml) were dialysed for at least 50 h before analysis.

The concentrations of samples for all physical measurements were determined after dialysis and dilution by sialic acid measurements, with a stock solution of known concentrations of the mucins as a standard (see below).

Preparation of mucins (Scheme 1)

Pregnancy mucus spontaneously released just before delivery was collected by specially instructed midwives. Only specimens that were coherent, that were not obviously contaminated (except for a small amount of blood) and that could be frozen immediately were used. Samples were stored at −20°C.

Three or four specimens were pooled (approx. 10 g) according to blood group. The mucus gel was gently stirred with di-isopropyl phosphorofluoridate (2.5 ml of a 0.1 M solution in propanol) for 5 min before the addition of ice-cooled 6 M-guanidinium chloride/5 mM-Na_2EDTA/5 mM-*N*-ethylmaleimide/10 mM-sodium phosphate buffer, pH 6.5 (120 ml). After dispersion (two or three strokes; Dounce homogenizer, loose pestle), the mucus was stirred for approx. 15 h at 4°C. Insoluble material was removed



Scheme 1. *Extraction, purification and yields of mucins from cervical mucus*

For full experimental details see the text. The yield is expressed as mg of non-diffusible freeze-dried solids/g of mucus (wet weight), and the distribution of this material is given as percentages (w/w) of the total. Numbers in parentheses show the distribution of mucins between the major fractions expressed as percentages (w/w) of high-molecular-weight glycopeptides obtained after papain/nuclease digestion and chromatography on Sepharose CL-4B.

by centrifugation (18000 rev./min for 60 min; Sorvall, SS-34 rotor) and re-extracted twice (approx. 20 ml of the extractant, 2 h at 4°C). CsCl and 5 mM-Na_2EDTA/10 mM-sodium phosphate buffer, pH 6.5, were added to a density of approx. 1.39 g/ml in 4 M-guanidinium chloride, and isopycnic density-gradient centrifugation was performed with the conditions specified in the legend to Fig. 1. This step was repeated (see Scheme 1), and the mucins (fraction IIb) were dialysed against 0.2 M-guanidinium chloride / 5 mM-Na_2EDTA / 10 mM-sodium phosphate buffer, pH 6.5. CsCl was added to a density of 1.50 g/ml, and a third density-gradient centrifugation step was performed as described

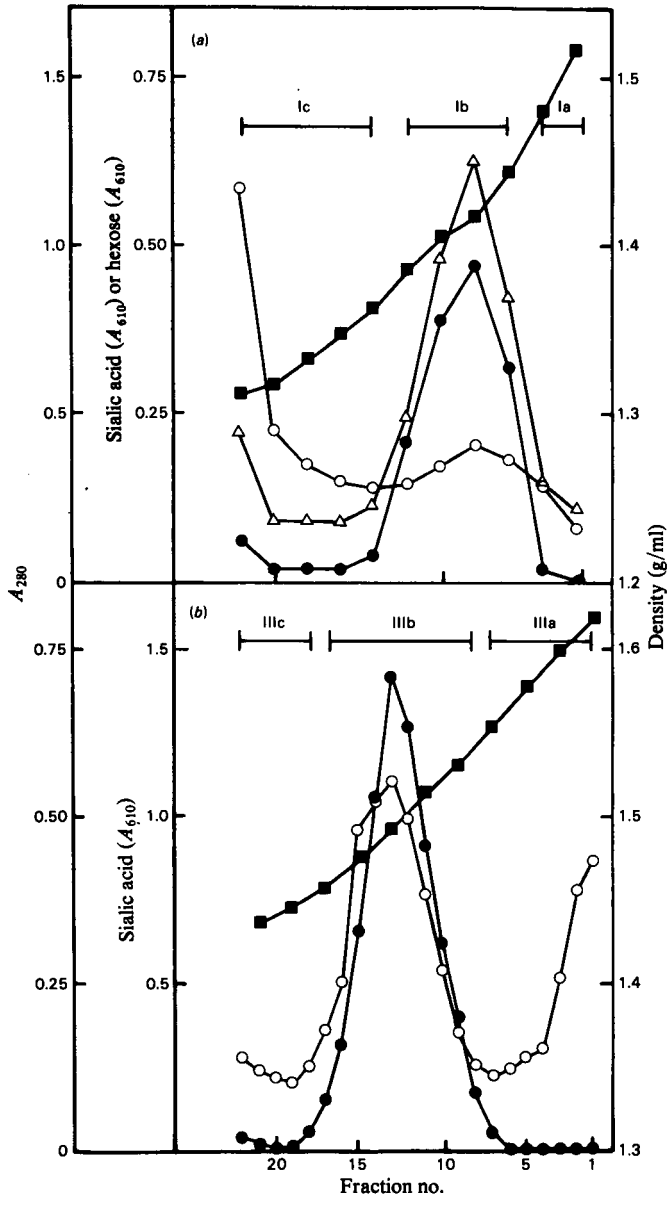


Fig. 1. *CsCl-density-gradient centrifugation of mucins in 4 M-guanidinium chloride (a) and 0.2 M-guanidinium chloride (b)* (*a*) After centrifugation (approx. 65 h; 36000 rev./min; MSE 65 ultracentrifuge; 8 × 25 ml rotor; 15°C), fractions were collected from the bottom of the tubes and analysed for sialic acid (●), hexose (△), A_{280} (○) and density (■). Fractions were pooled as shown by the horizontal bars. (*b*) Mucins twice purified by density-gradient centrifugation in CsCl/4 M-guanidinium chloride were subjected to density-gradient centrifugation in 0.2 M-guanidinium chloride and analysed as described above.

above. The final mucin preparation (fraction IIIb) was dialysed against 4 M-guanidinium chloride, pH 7, and stored frozen or in the cold. The concentration of this stock solution was assessed gravimetrically after extensive dialysis and freeze-drying of a sample. Side-fractions were recovered by freeze-

drying after dialysis. For a summary of the preparation and for information on the yield of the various fractions see Scheme 1.

The amount of CsCl added to CsCl/guanidinium chloride gradients was calculated from the equation

$$x = v(1.347\rho - 0.0318M - 1.347)$$

where x is CsCl (g), v is the final volume (ml), M is the molarity of guanidinium chloride and ρ is the density (g/ml).

Isolation of high-molecular-weight glycopeptides

Purified mucins and side-fractions were digested with papain in 1 M-NaCl/5 mM-Na_2EDTA/5 mM-cysteine hydrochloride/10 mM-sodium phosphate buffer, pH 7.0 for 15 h at 65°C followed by digestion with deoxyribonuclease and ribonuclease in 10 mM-$MgCl_2$/10 mM-sodium phosphate buffer, pH 7.0, for 15 h at 37°C. The glycopeptides were recovered after gel chromatography on Sepharose CL-4B as described in the legend to Fig. 2.

Results

Isolation and purification of cervical mucins

The mucus gel readily dissolved in 6 M-guanidinium chloride, leaving about 5–10% (w/w) of the total as an insoluble residue. The result of the first preparative density-gradient centrifugation in 4 M-guanidinium chloride is shown in Fig. 1(*a*). The mucins (fraction Ib, Scheme 1) were recovered between the densities 1.38 g/ml and 1.48 g/ml, whereas the bulk of the proteins were found at the top of the gradient (fraction Ic). Most of the residual proteins were removed by the second centrifugation step. When the fraction IIb was centrifuged in CsCl/0.2 M-guanidinium chloride, the mucins banded at a much higher density (between 1.45 g/ml and 1.55 g/ml) than in 4 M-guanidinium chloride. In addition, the presense of a u.v.-absorbing component devoid of sialic acid was now evident at a density of about 1.6 g/ml. This component showed a u.v.-absorption spectrum characteristic of that of DNA (results not shown).

The purified mucins and the major protein fractions (Ic and IIc) represented approx. 35% (w/w) and 50% (w/w) respectively of the total non-diffusible freeze-dried solids. This corresponds to about 1.5% (w/w) of mucin and 2.3% (w/w) of protein of mucus (wet weight). The glycopeptides derived from the purified mucins (fraction IIIb) and from the extraction residue showed similar chromatograms on Sepharose CL-4B, whereas most of the glycopeptides from the major protein fraction (Ic) were much more retarded (Fig. 2). The major glycopeptide peak from the purified mucins and corresponding fractions from fraction Ic and the extraction residue were pooled and recovered. The relative yields of such glycopeptides show that the purified mucins constitute approx. 85% of the total.

No proteins could be detected by polyacrylamide-gel electrophoresis of the purified mucins (0.5 mg of mucin/gel). Furthermore, when a sample of purified mucins (10 mg) was re-run in CsCl/4 M-guanidinium chloride, no proteins could be discerned when the whole top fraction was subjected to polyacrylamide-gel electrophoresis (results

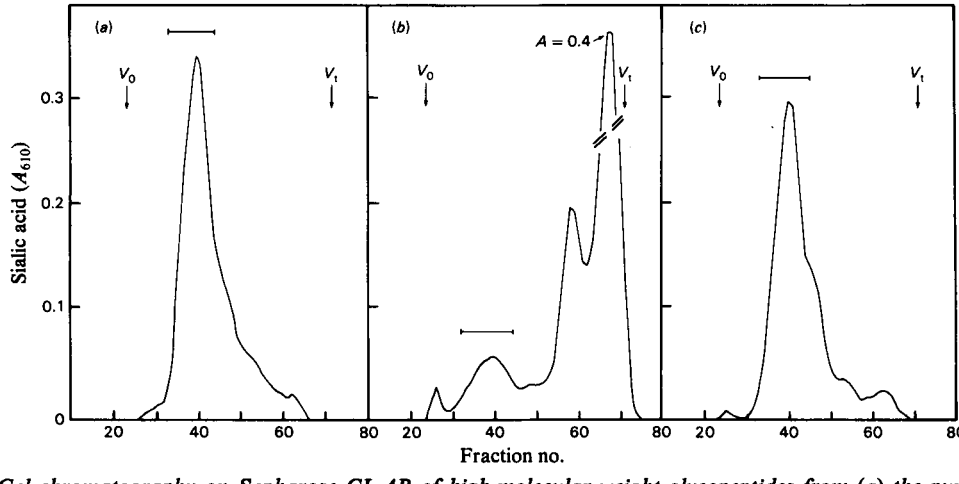


Fig. 2. *Gel chromatography on Sepharose CL-4B of high-molecular-weight glycopeptides from (a) the purified mucins (fraction IIIb), from (b) fraction Ic and from (c) the extraction residue*

Glycopeptides obtained after papain/nuclease digestion were chromatographed on a column (13.5 mm × 1486 mm) equilibrated with 4 M-guanidinium chloride/1 mM-Na_2EDTA/1 mM-sodium phosphate buffer, pH 6.5 (flow rate approx. 7 ml/h). Fractions (approx. 3 ml) were analysed for sialic acid and pooled as shown by the bars.

not shown). The extent of purification was also monitored by analytical isopycnic density-gradient centrifugation. Patterns of the crude mucin extract and of the mucin fractions after each stage of purification are shown in Fig. 3. Most of the proteins were removed by the first density-gradient centrifugation step (cf. Figs. 3*a* and 3*b*). The position of the sharp band at the high-density end of the mucin distribution corresponds to that of DNA under these conditions (results not shown). No proteins could be detected at the meniscus after the second purification step (Fig. 3*c*), and no DNA could be discerned in the mucin band after the third one (Fig. 3*d*). A u.v.-absorption spectrum of the purified mucins did not reveal the presence of any nucleic acids (results not shown).

When the effect of the guanidinium chloride concentration on the buoyant density of the mucins and of DNA in CsCl density gradients was studied further, it was noted that both macromolecules show a decrease in buoyant density as the concentration of guanidinium chloride is increased (Fig. 4). The difference in buoyant density between DNA and mucin was much larger in CsCl alone (about 0.18 g/ml) than in CsCl/4 M-guanidinium chloride (about 0.05 g/ml).

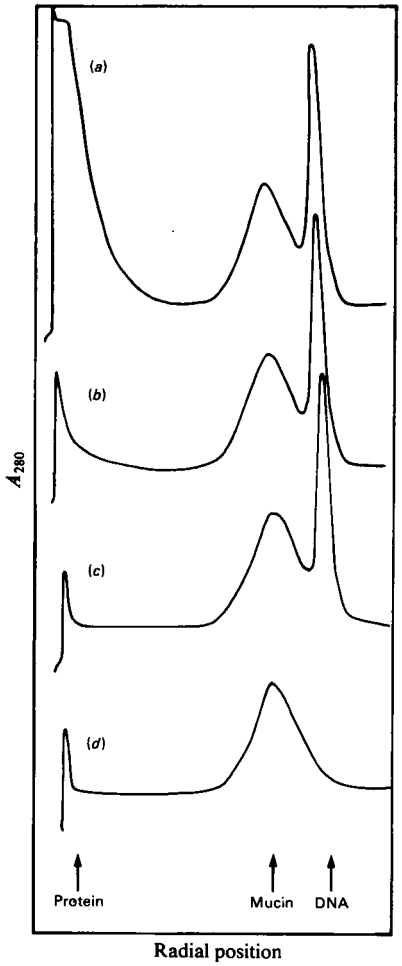


Fig. 3. *Analytical isopycnic density-gradient centrifugation of cervical mucins at various stages of purification*

(*a*) Crude mucin extract; (*b*) and (*c*) mucins purified once (fraction Ib) and twice (fraction IIb) respectively in CsCl/4 M-guanidinium chloride; (*d*) final mucin preparation obtained after the third purification step in CsCl/0.2 M-guanidinium chloride (fraction IIIb). Samples (approx. 0.5 mg of mucin/ml) were dialysed into CsCl/4 M-guanidinium chloride (ultrapure), initial density approx. 1.42 g/ml. Centrifugation was performed at 55 000 rev./min for 40 h at 25°C and the absorbance optics were used at 280 nm.

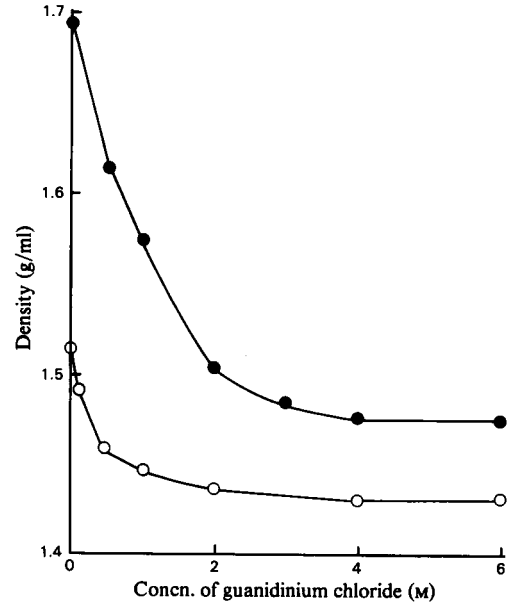


Fig. 4. *Buoyant density of cervical mucins and of DNA in CsCl/guanidinium chloride as a function of the guanidinium chloride concentration*

Cervical mucin and DNA (about 1 mg and 0.1 mg respectively) were dissolved in the molarity of guanidinium chloride under study, and CsCl was added to an initial density close to the expected buoyant density. After centrifugation (see the legend to Fig. 1), fractions were collected from the bottom and analysed for sialic acid and for absorbance at 280 nm (DNA). The density of the peak fraction was measured in each experiment and was plotted against the molarity of guanidinium chloride. ●, DNA; ○, cervical mucin.

Physical and chemical characterization of the mucins

The mucins chromatographed with the void volume of a Sepharose CL-2B gel in 6 M-guanidinium chloride, and appeared as a single unimodal peak (results not shown) when studied by sedimentation-velocity centrifugation in 0.2 M-NaCl as well as in 6 M-guanidinium chloride in the analytical ultracentrifuge. Zimm plots of data for mucins in 0.2 M-NaCl and 6 M-guanidinium chloride are shown in Figs. 5(*a*) and 5(*b*) respectively. A molecular weight of 5.9×10^6 was found in 6 M-guanidinium chloride and a value of 9.7×10^6 in 0.2 M-NaCl. In 6 M-guanidinium chloride, however, the mucins have a somewhat higher radius of gyration (197 nm) than in 0.2 M-NaCl (168 nm). Molecular weights obtained in 0.2 M-KCl and 0.066 M-$CaCl_2$ were close to those observed in 0.2 M-NaCl, whereas the cognate radii of gyration were somewhat larger. The molecular weights (6 M-guanidinium chloride) of mucins from fraction IIb prepared in the presence as well as in the absence of di-isopropyl phosphorofluoridate and *N*-ethylmaleimide were 7.9×10^6 and 7.2×10^6 respectively. For a summary of the physical data see Table 1.

It should be noted that light-scattering measurements could be influenced by extraneous large particles. In particular, when working with glycoproteins from mucus, the possibility of micro-gel formation or the presence of small amounts of very large aggregates must be considered, as they would make a considerable contribution to the molecular weight and the radius of gyration. To test this possibility, mucin solutions were subjected to ultrafiltration on 0.45 μm and 1.2 μm filters. The mucins did not penetrate the 0.45 μm filter at all. About one-third of a solution of mucins in 6 M-guandinium chloride was filtered through a 1.2 μm filter and diffusate as well as retained filtration residue were studied by light-scattering. The same molecular weight and radius of gyration were found for mucins that passed through and mucins that were retained, showing that there was no enrichment of larger particles above the filter. Thus the molecular weights do not seem to be overestimated because of the presence of aggregates. However, the radius of gyration of the mucins approaches the upper limit available with a conventional light-scattering apparatus, where readings are not possible below 30°C (see, e.g., Tanford 1961). If the limiting slope of the line $\theta \rightarrow 0$, at $c = 0$ (the so-called particle scattering function), is not reached, the molecular weight and the radius of gyration could be underestimated rather than overestimated (see also Eisenberg, 1971).

The chemical composition of the mucins is shown in Tables 2 and 3. The macromolecules contain approx. 20% (w/w) of protein, and 65% (w/w) is accounted for as carbohydrate. Serine and threonine comprise about 32 mol/100 mol of the amino acids. The mucins are low in aromatic amino acids. Tryptophan, however, is destroyed under the conditions used for acid hydrolysis, and cannot be evaluated by this method. The glucosamine/galactosamine molar ratio was between 1.22:1 and 1.46:1 for the various preparations studied. Galactose was

Table 1. *Summary of physical data for cervical mucin*
Values are means ± S.D., with numbers of preparations given in parentheses. All solvents include 1 mM-Na_2EDTA/1 mM-sodium phosphate buffer, pH 6.5. R_G is the radius of gyration and d*n*/d*c* is the refractive-index increment.

Conditions	$10^{-6} \times M_w$	R_G (nm)	d*n*/d*c* (ml/g)	$s_{20,w}$ (S)
0.2 M-NaCl	9.7 ± 0.35 (3)	168 ± 14 (3)	0.172	50.1
0.2 M-KCl	9.6 (1)†	199 (1)		
0.066 M-$CaCl_2$*	9.6 (1)†	182 (1)		
6 M-Guanidinium chloride	5.9 ± 0.56 (6)	197 ± 18 (6)	0.135	37.0

* EDTA and sodium phosphate were omitted from this solution.

† The value for d*n*/d*c* in 0.2 M-NaCl was used.

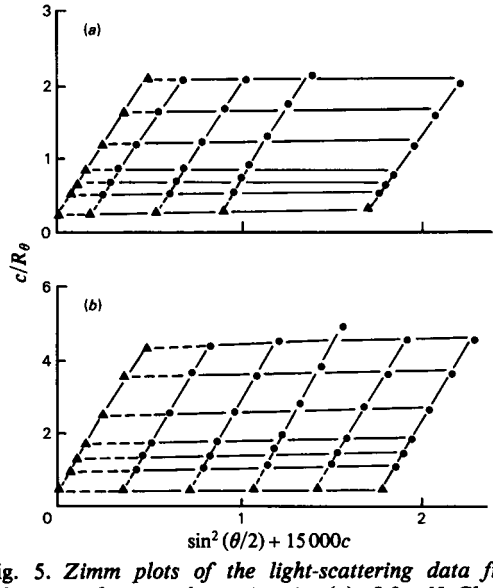


Fig. 5. *Zimm plots of the light-scattering data from solutions of cervical mucins in (a) 0.2 M-NaCl and (b) 6 M-guanidinium chloride*
The following concentrations were used: (*a*) 0.012, 0.024, 0.036 and 0.111 mg/ml; (*b*) 0.024, 0.048, 0.072, 0.096 and 0.199 mg/ml. The optical constants were (*a*) 4.636×10^{-7} cm$^2 \cdot$g^{-2} and (*b*) 3.56×10^{-7} cm$^2 \cdot$ g^{-2}.

Table 2. *Chemical composition of cervical mucin*
Values are means ± S.D. for five different preparations. Amino sugars were determined by using the amino acid analyser and neutral sugars by g.l.c. Galactose was also determined by the anthrone method. As fucose gives approx. a 50% higher colour yield than galactose by this method, a correction was made by using data for fucose from g.l.c. Glucosamine, *N*-acetylneuraminic acid and protein (bovine serum albumin and ribonuclease) do not interfere. Sialic acid is expressed as *N*-acetylneuraminic acid, and protein is the sum of the values for the individual amino acids. All numbers are corrected for moisture (approx. 9%).

	Composition	
	(g/100 g of mucin)	(molar proportions of monosaccharides)
N-Acetylgalactosamine	9.7 ± 0.8	1.00
N-Acetylglucosamine	12.8 ± 0.4	1.32
Galactose*	20.6 ± 0.9	2.61
Galactose†	18.7 ± 0.8	2.37
Fucose	7.5 ± 1.1	1.04
Sialic acid	15.0 ± 1.0	1.11
Protein	19.9 ± 0.8	

* By the anthrone method.
† By g.l.c.

Table 3. *Amino acid composition of cervical mucins*
All values are means ± S.D. for five different preparations. Cysteine was determined after performic acid oxidation (Hirs, 1967).

Amino acid	Composition (mol/1000 mol of total amino acids)
Asx	53.1 ± 1.0
Thr	207.1 ± 7.4
Ser	114.5 ± 3.5
Glx	71.9 ± 4.6
Pro	104.4 ± 2.6
Gly	79.7 ± 1.9
Ala	89.1 ± 1.7
CyS	30.3 ± 1.5
Val	51.5 ± 0.6
Met	4.5 ± 1.1
Ile	21.6 ± 0.5
Leu	58.7 ± 1.0
Tyr	14.8 ± 1.0
Phe	21.4 ± 1.6
His	23.3 ± 0.9
Lys	20.2 ± 1.0
Arg	34.2 ± 0.8

the major neutral sugar found, whereas mannose and glucose were present in trace amounts [less than 0.5% (w/w) of the mucins].

Discussion

The object of this work is to establish a protocol for the isolation of undegraded mucins. Potentially destructive methods such as sonication and high-speed homogenization were thus avoided for the solubilization of the mucus gel. High-speed homogenization apparently does not degrade gastric mucins (Robson *et al.*, 1975). In contrast, cartilage proteoglycans are markedly degraded by this treatment (Sajdera & Hascall, 1969). Sonication is used as a routine to induce fragmentation of high-molecular-weight DNA (see, e.g., Godfrey, 1976). Modification of the mucins by enzymes present in mucus is a distinct possibility that, in our opinion, has not been given enough consideration in the past. It has, for example, been shown that pig gastric mucus contains a neuraminidase (Allen & Starkey, 1974) and human cervical mucus an α-L-fucosidase (Chantler & Debruyne, 1977). The probable presence of proteolytic enzymes in mucus has also been pointed out (see, e.g., Gibbons & Sellwood, 1973).

To protect the mucins from degradative enzymes during preparation, we used guanidinium chloride supplemented with proteinase inhibitors as extractant. Di-isopropyl phosphorofluoridate and EDTA were used to inhibit serine proteinases and metalloproteinases respectively. *N*-Ethylmaleimide served the dual function of inhibiting proteinase containing a thiol group in the active centre and of blocking, if present, free thiol groups on the mucins that could otherwise participate in thiol–disulphide exchange reactions that are favoured under highly denaturating conditions (Tanford, 1968; Creeth, 1977). By such reactions extraneous proteins might be attached to the mucins and/or internal disulphide bridges might become scrambled. A pH below 7 should also act to decrease this possibility (Tanford, 1968).

Isopycnic density-gradient centrifugation in caesium salts has been successfully used for the purification of mucins from many sources. For a review see, for example, Creeth (1978). As this procedure can be conducted in the presence of high concentrations of guanidinium chloride, removal of proteins, and thus degradative enzymes, can be achieved under conditions that should suppress enzymic activity.

The complete removal of proteins from the purified mucins was assessed by three criteria: (*a*) polyacrylamide-gel electrophoresis, (*b*) analytical density-gradient centrifugation and (*c*) re-running the purified mucins in a CsCl/4 M-guanidinium chloride density gradient. The absence of nucleic acids was evident from analytical density-gradient centrifugation and by u.v. spectroscopy.

Mucins and DNA showed a much lower buoyant density in CsCl/4 M-guanidinium chloride than in CsCl alone. This could be explained by a lower Cs^+

binding to negative groups due to competition by the guanidinium ion and/or a higher degree of hydration of the macromolecules in 4M-guanidinium chloride. The difference in buoyant density between the mucins and DNA increased as the concentration of guanidinium chloride was decreased. This explains why mucins and DNA could be separated in CsCl/0.2M-guanidinium chloride whereas no separation was achieved in CsCl/4M-guanidinium chloride.

The mucins appeared as a unimodal, although polydisperse, distribution in sedimentation-velocity centrifugation and analytical density-gradient centrifugation, and no heterogeneity was evident. The macromolecules are thus homogeneous in size and buoyant density by the criteria suggested by Gibbons (1963). The difference in molecular weights in 6M-guanidinium chloride and in 0.2M-NaCl suggested that a limited aggregation takes place when the mucins are brought from denaturating conditions into a solution with a physiological ionic strength. This aggregation is fully reversible and was observed in all preparations studied. There is no concomitant decrease in the radius of gyration, suggesting that dissociation is accompanied by an expansion of the individual macromolecule in 6M-guanidinium chloride. It should be pointed out that the molecular weight is very sensitive to the value of the refractive-index increment (dn/dc), as this parameter is entered squared into the equation. The determination of dn/dc in 6M-guanidinium chloride is difficult, in particular because the physical nature of the macromolecules precludes measurement at concentrations much above 3mg/ml. However, the lower sedimentation coefficient in guanidinium chloride suggests that dissociation of aggregates and/or a conformational change takes place when the mucins are dialysed into this solvent. The mucins sediment as single peaks in guanidinium chloride as well as in NaCl, suggesting that the bulk of the material is affected.

The molecular weight of mucins from fraction IIb (Scheme 1) was somewhat higher (7.2×10^6) than that of the finally purified ones. We do not know, at present, whether this difference is an expression of the variability between preparations or could be explained by a slight contamination with high-molecular-weight DNA. These observations show, however, that no irreversible aggregation occurs when the mucins are dialysed from 6M-guanidinium chloride into 0.2M-guanidinium chloride (see, e.g., Snary *et al.*, 1974). The molecular weight of mucins from fraction IIb prepared in the absence of di-isopropyl phosphorofluoridate and *N*-ethylmaleimide was 7.9×10^6, indicating that these proteinase inhibitors do not cause any increase of the molecular weight.

The molecular weights and the sedimentation coefficients correspond to frictional ratios for the mucins of 4.5 and 4.6 in 6M-guanidinium chloride and 0.2M-NaCl respectively. These high frictional ratios could be explained by either a highly elongated structure or a large degree of hydration, e.g. the mucins behave as flexible chains entrapping a lot of solvent. The latter description fits well with that of Lee *et al.* (1977*a*,*b*). Those authors suggest that cervical mucus is composed of a heavily entangled network of macromolecules with molecular wights about 10×10^6. Furthermore, the first macromolecular disperse entity that dissolves from bovine cervical mucus was found by light-scattering to have a molecular weight of 15×10^6 and a radius of gyration of approx. 200nm (Meyer & Silberberg, 1978). Finally, Gibbons & Glover (1959), using flow-birefringence, described bovine cervical mucins as long flexible chains with molecular weights of approx. 4×10^6.

The chemical composition of the cervical mucins described in the present paper is typical of that of mucus glycoproteins in general. The amino acid composition is similar to those reported by Wolf *et al.* (1980) and by Yurewicz & Moghissi (1981) for human cervical mucins and by Hatcher *et al.* (1977) for cervical mucins from the bonnet monkey. The rather high protein content reported by Iacobelli *et al.* (1971) for human pregnancy mucins is probably explained by the fact that these authors used gel chromatography alone to purify the mucins. The carbohydrate analyses are consistent with those found for mucins from many sources (Clamp *et al.*, 1978; see also Meyer *et al.*, 1977). In the present study the glucosamine content was consistently higher than that of galactosamine. This conforms with Wolf *et al.* (1980) but is at variance with, e.g., Hatcher *et al.* (1977), Yurewicz & Moghissi (1981) and Van Kooij *et al.* (1980). For a summary of compositional analyses of cervical mucins see Chantler (1982).

No significant differences in carbohydrate analyses were discerned between mucins from donors belonging to different blood-groups within the ABO system. However, as the secretor status was not established, and as mucus from several women provided the starting material for each preparation, the predicted differences could very well have been averaged out. Furthermore, no differences in carbohydrate analyses were found by Van Kooij *et al.* (1980) in a study of cervical mucins from women belonging to different blood-groups.

It is concluded that the present protocol gives rise to highly purified mucins of high molecular weight and with little variation between preparations. The 'native' conformation of the mucins could, however, very well have been perturbed by the high concentrations of CsCl and guanidinium chloride used (Snary *et al.*, 1974). The isolation of a mucin that

has not been modified by the breakage of covalent bonds is a prerequisite for structural studies, and should also provide a reference point for the development of alternative protocols for the isolation of mucins that employs more physiological solvent conditions.

This investigation was supported by grants from the Swedish Medical Research Council (567 and 5637; Visiting Scientist Fellowship for J. K. S.) and the Medical Faculty of Lund. Valuable discussions and criticism by Dr. Lars Cöster, Dr. Lars-Åke Fransson and Dr. Anders Malmström are gratefully acknowledged.

References

Allen, A. & Starkey, B. J. (1974) *Biochim. Biophys. Acta* **338**, 364–368

Bhaskar, K. R. & Creeth, J. M. (1974) *Biochem. J.* **143**, 669–679

Carlstedt, I., Sheehan, J., Ulmsten, U. & Wingerup, L. (1982) in *Mucus in Health and Disease II* (Chantler, E., Elder, J. & Elstein, M., eds.), pp. 273–275, Plenum Press, New York

Casassa, E. F. & Eisenberg, H. (1964) *Adv. Protein Chem.* **19**, 287–395

Chantler, E. (1982) in *Mucus in Health and Disease II* (Chantler, E., Elder, J. & Elstein, M., eds.), pp. 251–263, Plenum Press, New York

Chantler, E. & Debruyne, E. (1977) in *Mucus in Health and Disease* (Elstein, M. & Parke, D. V., eds.), pp. 131–141, Plenum Press, New York

Chretien, F. C., Engelman, P. & Dubois, R. (1979) *Eur. J. Obstet. Gynecol. Reprod. Biol.* **9**, 289–297

Clamp, J. R., Allen, A., Gibbons, R. A. & Roberts, G. P. (1978) *Br. Med. Bull.* **34**, 25–41

Creeth, J. M. (1977) *Mod. Probl. Paediatr.* **19**, 34–45

Creeth, J. M. (1978) *Br. Med. Bull.* **34**, 17–24

Eisenberg, H. (1971) *Proced. Nucleic Acid Res.* **2**, 137–175

Elstein, M. (1982) in *Mucus in Health and Disease II* (Chantler, E., Elder, J. & Elstein, M., eds.), pp. 301–318, Plenum Press, New York

Gibbons, R. A. (1963) *Nature (London)* **200**, 665–666

Gibbons, R. A. & Glover, F. A. (1959) *Biochem. J.* **73**, 217–225

Gibbons, R. A. & Sellwood, R. (1973) in *The Biology of the Cervix* (Blandau, R. J. & Moghissi, K., eds.), pp. 251–265, University of Chicago Press, Chicago

Goa, J. (1955) *Scand. J. Clin. Lab. Invest.* **7**, *Suppl.* **22**, 19–25

Godfrey, J. E. (1976) *Biophys. Chem.* **5**, 285–299

Hatcher, V. B., Schwartzmann, G. O. H., Jeanloz, R. W. & McArthur, J. W. (1977) *Biochemistry* **16**, 1518–1524

Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 59–62

Iacobelli, S., Garcea, N. & Angeloni, C. (1971) *Fertil. Steril.* **22**, 727–734

Jourdian, G. W., Dean, L. & Roseman, S. (1971) *J. Biol. Chem.* **246**, 430–435

Lee, W. I., Blandau, R. J. & Verdugo, P. (1977*a*) in *The Uterine Cervix in Reproduction* (Insler, V. & Bettendorf, G., eds.), pp. 68–77, Georg Thieme, Stuttgart

Lee, W. I., Verdugo, P., Blandau, R. J. & Gaddum-Rosse, P. (1977*b*) *Gynecol. Invest.* **8**, 254–266

Lindahl, U. (1970) *Biochem J.* **116**, 27–34

Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H. & Heinegård, D. (1980) *J. Biol. Chem.* **255**, 6084–6091

Meyer, F. A. & Silberberg, A. (1978) *Respiratory Tract Mucus: Ciba Found. Symp. (New Ser.)* **54**, 203–211

Meyer, F. A., Vered, J. & Sharon, N. (1977) in *Mucus in Health and Disease* (Elstein, M. & Parke, D. V., eds.), pp. 239–249, Plenum Press, New York

Neville, D. N. (1971) *J. Biol. Chem.* **246**, 6328–6334

Robson, T., Allen, A. & Pain, R. H. (1975) *Biochem. Soc. Trans.* **3**, 1105–1107

Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87

Snary, D., Allen, A. & Pain, R. H. (1974) *Biochem. J.* **141**, 641–646

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, pp. 297–316, John Wiley and Sons, New York

Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121–282

Tomimatsu, Y., Vitello, L. & Fong, K. (1968) *J. Colloid Interface Sci.* **27**, 573–580

Van Kooij, R. J., Roelofs, H. M. J., Kathmann, G. A. M. & Kramer, M. F. (1980) *Fertil. Steril.* **34**, 226–233

Wolf, D. P., Blasco, L., Khan, M. A. & Litt, M. (1978) *Fertil. Steril.* **30**, 163–169

Wolf, D. P., Sokoloski, J. E. & Litt, M. (1980) *Biochim. Biophys. Acta* **630**, 545–558

Yurewicz, E. C. & Moghissi, K. S. (1981) *J. Biol. Chem.* **256**, 11895–11904

Zimm, B. H. (1948) *J. Chem. Phys.* **16**, 1099–1116