Biochem. J. (1979) **181**, 725–732
Printed in Great Britain

The Role of Disulphide Bonds in Human Intestinal Mucin

By Janet F. FORSTNER,*†‡ Inderjit JABBAL,* Rauf QURESHI,* David I. C. KELLS† and Gordon G. FORSTNER*

**Research Institute, The Hospital for Sick Children, Toronto, Ont., Canada M5G 1X8, and †Department of Biochemistry, University of Toronto, Toronto, Ont., Canada M5S 1A8*

(*Received* 12 *February* 1979)

Goblet-cell mucin (mucin 1) was isolated and purified from human small-intestinal scrapings. After application of mucin 1 to DEAE-Bio-Gel (A) columns, most of the glycoprotein (76–94% of hexoses) was eluted in the first peak (designated mucin 2). Minor amounts of acidic glycoproteins were eluted with 0.2M- and 0.4M-NaCl in later peaks. Analyses of mucin 1 and mucin 2 revealed mucin 2 to be a monodisperse highly glycosylated glycoprotein containing 6.3% by wt. of protein, *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose and fucose. Mucin 1 was similar in composition, but was polydisperse and contained more protein (12.3% by wt.) as well as *N*-acetylneuraminic acid. Analytical CsCl-gradient ultracentrifugation showed both mucin 1 and mucin 2 to have a major component with an average buoyant density of 1.47000g/ml. Mucin 1 also contained a slightly less-dense minor glycoprotein component. After exhaustive reduction and alkylation mucin 1 retained its major component, but partly dissociated into two lighter glycoprotein components. Mucin 2, in contrast, did not change its density distribution after reduction. Band ultracentrifugation in ^2H_2O-containing iso-osmotic buffers showed that mucin 1 contained a major fast-sedimenting component ($s^0 = 37 \pm 2$S), and a minor amount of a slower-sedimenting component. After reduction there was an increased quantity of the latter component, for which an s^0 value of 14.5S was calculated. In contrast, mucin 2 was unaltered by reduction ($s^0 = 33 \pm 2$S). These findings indicate that the major component of goblet-cell mucin (mucin 2) does not dissociate after S–S-bond reduction, and thus does not apparently rely for its polymeric structure on the association of subunits through covalent disulphide bonds. However, the effects of reduction on mucin 1 suggest that in the native mucin intramolecular disulphide bonds in the minor glycoproteins may stabilize their structure, permitting secondary non-covalent interactions to develop with the major dense mucin (mucin 2) protein.

Over the past 5–10 years several studies carried out on mucus glycoproteins from many organs have suggested that these macromolecules consist of subunits held together by interchain disulphide bonds and further stabilized by non-covalent interactions (Dunstone & Morgan, 1965; Allen & Snary, 1972; Bhaskar & Creeth, 1974; Oemrawsingh & Roukema, 1974; Roberts, 1974, 1976; Starkey *et al.*, 1974; Robinson & Monsey, 1975; Robson *et al.*, 1975; Creeth *et al.*, 1977; Marshall & Allen, 1978). The end result of multiple interconnections is an extended and random gel network, which imparts to mucus secretions their characteristic property of viscoelasticity. Evidence that S–S bonds play an important structural role has been provided by demonstrations that thiol-group reagents decrease the viscosity and increase the solubility of native mucus secretions (Roberts, 1974, 1976), and in some cases decrease the molecular weight of purified mucins. For example, Starkey *et al.* (1974) observed that the sedimentation coefficient of a large porcine gastric mucin decreased from 33S (mol.wt. 2.3×10^6) to 14S (mol.wt. 2.5×10^5) after treatment with 0.2M-2-mercaptoethanol. Somewhat similar but less dramatic effects have been observed after reduction of bronchial mucus glycoproteins (Creeth *et al.*, 1977; Roberts, 1976), cervical mucins (Gelman & Vered, 1976; Masson, 1973) and egg-white β-ovomucin (Robinson & Monsey, 1975).

Despite these observations, there remain some difficulties in accepting a central role for S–S bonds in the molecular architecture of all mucin molecules. Firstly, highly purified mucins characteristically have a very low content of cysteine. In purified ovine submaxillary mucin, there are no cysteine residues at all (Hill *et al.*, 1977), and no evidence of dissociation by -SH agents (Hill *et al.*, 1977; Holden *et al.*, 1971).

Abbreviation used: SDS, sodium dodecyl sulphate.

‡ To whom reprint requests should be addressed at: The Hospital for Sick Children, 555 University Avenue, Toronto, Ont., Canada M5G 1X8.

In bronchial mucins, Roussel *et al.* (1978) and Roberts (1976) found that the disulphide bridges cleaved by reducing agents belong mainly to the protein fraction of the mucus rather than the glycoprotein fraction. One explanation of these anomalies is that mucins may normally be polymerized through specific cross-linking peptides, which are enriched in cysteine and form covalent disulphide bonds with mucin cysteine residues. The linkage peptides are thought to exist either as specialized non-glycosylated areas of the peptide core of mucin glycoproteins (Allen *et al.*, 1974; Starkey *et al.*, 1974) or as discrete peptides which intertangle with glycosylated mucin fibres and form S–S bonds with them (Roberts, 1976). Another possibility that should be considered is that some mucin or glycopeptide molecules, because their three-dimensional structure is stabilized by intramolecular S–S bonds, are able to interpenetrate each others' molecular domains (Morris & Rees, 1978), producing secondary strong non-covalent cross-linking or aggregation into mucin polymers.

In the following study we assessed the effects of thiol reduction on the structure of two preparations of mucin obtained from human small-intestinal tissue. The two preparations, mucin 1 and mucin 2, differed with respect to their composition and polydispersity. They were analysed by ultracentrifugal techinques before and after reduction and alkylation of disulphide bonds. Special attention was given to the technique of analytical equilibrium centrifugation in CsCl density gradients, following methods outlined for mucus glycoproteins by Creeth *et al.* (1977) and Bhaskar & Creeth (1974). An effort was made to detect changes in buoyant density and polydispersity of the mucins, and to look for the liberation of potential cross-linking proteins after thiol reduction.

The more polydisperse preparation of mucin (mucin 1) gave evidence of partial dissociation after reduction, whereas the major mucin component (mucin 2) was virtually unresponsive to reducing agents. The significance of these findings for the formation and degradation of mucin polymers is discussed.

Experimental

Preparation of human goblet-cell mucin

Human intestine was obtained at autopsy within 5h *post mortem*. Sections of jejunum were examined histochemically by routine haematoxylin/eosin and periodic acid/Schiff base staining. The gross architecture of the mucosa was preserved and goblet cells appeared normal morphologically. Mucin was prepared from mucosal scrapings as described earlier (Jabbal *et al.*, 1979) by applying post-microsomal supernatant solutions to Sepharose 4B (Sigma) columns (1cm×90cm). The mucin was eluted in the void volume. Further purification was accomplished by pooling the void-volume peak, dialysing for 24h against three changes of distilled water, and centrifuging the non-diffusible component at 30000*g* for 30min at 4°C, thereby producing a protein-enriched pellet and a mucin-containing supernatant (mucin 1). After chemical and physical characterization of mucin 1, further fractionation was carried out on DEAE-Bio-Gel (A) (Bio-Rad, Richmond, CA, U.S.A.) columns (1cm×40cm) equilibrated with 50mM-Na_2HPO_4/NaH_2PO_4 buffer (pH6.0)/6M-urea. The column was sequentially developed with the same urea-containing buffer to which was added 0, 0.2M- and 0.4M-NaCl in a stepwise gradient. Fractions were dialysed and analysed for total hexoses (Spiro, 1966), *N*-acetylneuraminic acid (Svennerholm, 1957) and light absorption at 280nm. The first peak from this column contained most of the applied carbohydrate (ranging from 76 to 94%, average 85% in six separate experiments). It was pooled, dialysed exhaustively against distilled water, freeze-dried and resuspended in 2ml of water for subsequent analyses. Throughout this paper comparisons are made between the supernatant of the void volume of Sepharose 4B (i.e. mucin 1) and the major mucin component, mucin 2, which was eluted from DEAE-Bio-Gel as the first hexose peak.

SDS/polyacrylamide-disc-gel electrophoresis

This was carried out on 3.5% gels in 0.1% SDS with periodic acid/Schiff (PAS) and Coomassie Blue staining as described earlier (Jabbal *et al.*, 1976). Before application on the gels, mucin samples were incubated for 1h at 37°C in 1.0% SDS, a step found necessary for the demonstration of minor protein 'contaminants' during subsequent electrophoresis.

Amino acid analyses

These were performed on hydrolysates (6M-HCl, 22h, 110°C *in vacuo*) on a Durrum model 500 automatic amino acid analyser. Comparison of total amino acid content with values obtained by the method of Lowry *et al.* (1951) for protein revealed that the Lowry technique underestimated total protein by a factor of 1.57 when bovine serum albumin was used as a standard. All protein values obtained by this method were therefore appropriately corrected before presentation. Values of percentage of protein (per dry wt.) were obtained for freeze-dried samples of mucin dried to constant weight in a vacuum desiccator over P_2O_5.

Carbohydrate analyses

Carbohydrate components were determined by g.l.c. of the trimethylsilyl derivatives after methanolysis with 1M-HCl for 16h at 100°C (Clamp *et al.*, 1971; Chambers & Clamp, 1971). Silyl derivatives were applied to 3% SE-30 on chromasorb WHP

80–100 mesh columns (Chromatographic Specialties, Brockville, Ont., Canada) in a Varian aerograph g.l.c. series 2100 equipped with an automatic minigrator.

Analytical density-gradient ultracentrifugation in CsCl

A Beckman model E ultracentrifuge was used with schlieren optics. A stock solution of 42% (w/v) (or 43%) CsCl in phosphate buffer (33 mM-NaCl/16.7 mM-Na_2HPO_4/16.7 mM-NaH_2PO_4, pH 6.8) was prepared and the density obtained from refractive-index measurements (Chervenka, 1970). Sample solutions were prepared by mixing freeze-dried mucin with the stock CsCl solution to give a final concentration of 100 μg of mucin protein/ml. In some cases small portions (25 μg) of mucin samples were first stained with periodic acid/Schiff reagent (Jabbal *et al.*, 1975), and enough was added to CsCl to give absorbance readings at 555 nm of 2.0 absorbance units. Refractive indices and densities of the solutions were rechecked and then samples were added to double-sector cells (Kel-F-coated centrepieces). An An-H titanium rotor was used for obtaining schlieren optical patterns, and an An-F titanium rotor was used to obtain tracings of light absorption at 555 nm. Runs were made at 25°C at 44000 rev./min for 48 h. Peak heights were measured at 5 h intervals to ensure that equilibrium conditions were attained. Buoyant densities were calculated from schlieren optics or 555 nm tracings by using standard formulae (Chervenka, 1970).

Sedimentation-velocity studies of mucin 2 samples (0.3–1.39 mg/ml)

These were carried out in a Beckman model E ultracentrifuge at 20°C in double-sector cells as described earlier (Jabbal *et al.*, 1976). Sedimentation coefficients ($s^0_{20,w}$) were calculated by using schlieren optics, and the values were corrected for solvent density and viscosity.

Band ultracentrifugation

This was performed on mucin samples previously stained with periodic acid/Schiff reagent as described earlier (Jabbal *et al.*, 1975). Sedimentation coefficients were calculated from radial displacement of the peak maximal measured from spectrophotometric scans at 555 nm. This method was used more extensively than boundary-sedimentation velocity, because it avoids concentration-dependent alterations in sedimentation velocity, and gives reliable s^0 values with a single run. It could also be used on microgram quantities of glycoproteins that were in scarce supply.

Thiol reduction of mucin

Disulphide bond reduction of mucin samples was carried out in the presence of 6 M-guanidine hydrochloride and 0.01 M-dithiothreitol in 0.1 M-Tris/HCl, pH 8.5, followed by alkylation with 0.04 M-iodoacetamide. These conditions were found earlier (Forstner *et al.*, 1973) to reduce and alkylate all S–S bonds in rat intestinal mucin. Amino acid analyses performed on the mucin preparations before and after reduction and alkylation showed that all cysteine was converted to carboxymethylcysteine.

Results

The starting material for the mucin preparations in this study was intestinal tissue removed at autopsy (less than 5 h *post mortem*). Although minor chemical differences in the mucin product (mucin 1) were noted, it resembled another human preparation that we used earlier (Jabbal *et al.*, 1976) derived from a surgical specimen of intestine. The surgical and autopsy preparations gave a reaction of identity in

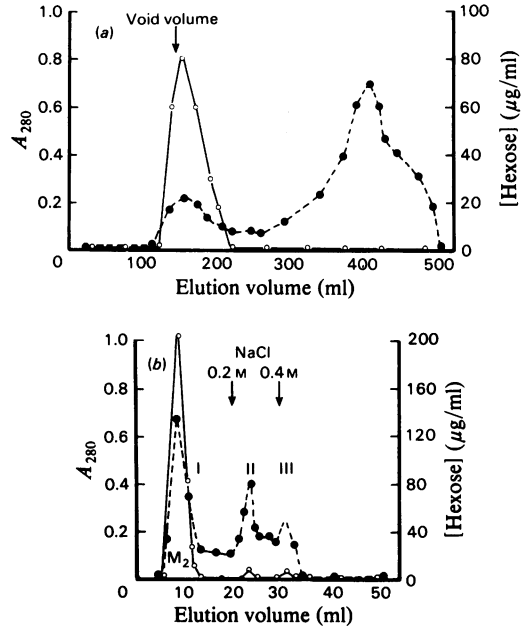

Fig. 1. *Preparation of human intestinal goblet-cell mucin*
(*a*) Post-microsomal cell sap was applied to a Sepharose 4B column (1 cm × 90 cm) and eluted with 0.1 M-NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0. Hexose (○) was measured with the anthrone reagent (Spiro, 1966) and protein (●) by the measuring light absorption at 280 nm. The void volume was determined with the aid of Blue Dextran. (*b*) The material in the void-volume peak from (*a*) was pooled, dialysed, concentrated and centrifuged at 30000*g* for 30 min. The supernatant (called mucin 1 in the text) was applied to a DEAE-Bio-Gel (A) column (1 cm × 40 cm) and eluted with 50 mM-Na_2HPO_4/NaH_2PO_4, pH 6.0, containing 6 M-urea. At indicated intervals, 0.2 M and 0.4 M-NaCl were included in the eluting buffer. Peak I is the major mucin species, called mucin 2 in the text.

double immunodiffusion against rabbit antisera to mucin (Jabbal *et al.*, 1979), and were almost identical in size and ultracentrifugal-sedimentation properties. It was judged therefore that the mucin used here was a satisfactory product for study, with no evidence of significant post-mortem degradation.

Figs. 1(*a*) and 1(*b*) show the chromatographic preparations of the mucin sample from jejunal tissue of a single patient. The material in the void-volume peak from Sepharose 4B (Fig. 1*a*) was harvested, dialysed, concentrated and centrifuged, as described in the Experimental section, to yield mucin 1. Subsequent fractionation of mucin 1 on DEAE-Bio-Gel (Fig. 1*b*) yielded the major mucin component mucin 2 (peak I). Small amounts of acidic glycoproteins were eluted as minor peaks (II, III). There was too little material in these peaks to permit full analysis, although colorimetric assays performed on both minor peaks gave positive reactions for sialic acid, hexoses and protein. Subsequent analyses throughout this paper are therefore confined to the two main mucin preparations, mucin 1 and the major component of mucin 1, designated mucin 2.

The amino acid profile and total protein content are shown in Table 1 for both mucin 1 and mucin 2 fractions. The two fractions had very similar profiles, the major difference being a higher serine content in mucin 2 than in mucin 1. There was also a slightly lower lysine, leucine and half-cystine content in mucin 2. No cysteic acid was detected in either preparation. The total protein content was only 6.3% by wt. in mucin 2, indicating that a significant loss of protein had resulted from DEAE-Bio-Gel chromatography of mucin 1 (13.3% of protein).

Table 2 shows the results of carbohydrate analyses. Compared with mucin 1 there was an enrichment in galactose and *N*-acetylglucosamine in mucin 2, with a decrease in *N*-acetylgalactosamine and sialic acid content. The overall hexose/protein ratio was much higher in mucin 2 (approx. 3-fold), indicating that it was much more highly glycosylated than the mucin 1 fraction. Neither preparation contained mannose. It appears therefore that poorly glycosylated glycoproteins containing *N*-acetylgalactosamine and sialic acid had been separated from the mucin 1 fraction during chromatography on DEAE-Bio-Gel.

The results of electrophoresis in 3.5% SDS/polyacrylamide disc gels are shown in Fig. 2. Staining with periodic acid/Schiff reagent for carbohydrate revealed in mucin 1 and mucin 2 a single broad band which barely entered the gels. This pattern is very characteristic of purified epithelial mucins (Holden *et al.*, 1971), reflecting their large size, asymmetric shape and poor diffusibility in gels. Coomassie Blue staining for protein revealed that the mucin 1, but not the mucin 2 preparation, contained five additional minor bands, suggesting that mucin 1 contained small amounts of poorly glycosylated or non-mucin peptides.

Mucin 1 and mucin 2 samples were further compared by analytical equilibrium ultracentrifugation in CsCl gradients before and after reduction and alkylation. Bhaskar & Creeth (1974), Creeth *et al.* (1977) and Creeth (1978) have shown that this technique has a high potential for the resolution of large glycoprotein species of different density, and is capable of detecting peptide contaminants even when

Table 1. *Amino acid analyses of chromatographic fractions of human goblet-cell mucin*

Mucin fractions were hydrolysed in 6M-HCl at 110°C for 22h and analysed on a Durrum model 500 automatic amino acid analyser. Each value is the average of two separate analyses, which gave almost identical results. Preparation of mucin 1 and mucin 2 is described in the text.

	Amino acid content (mol/100mol)	
	Mucin 1	Mucin 2
Asp	4.23	3.45
Thr	29.12	28.20
Ser	10.03	14.11
Glu	5.99	5.34
Pro	12.41	11.25
Gly	5.69	4.98
Ala	3.61	4.12
½Cys	0.98	0.42
Val	5.21	5.06
Met	0.22	0.14
Ile	3.72	4.09
Leu	5.37	3.80
Tyr	1.42	1.58
Phe	1.65	1.66
Lys	2.08	1.44
His	1.66	1.53
Arg	2.08	1.76
Total protein (% dry wt.)	13.3	6.3

Table 2. *Carbohydrate composition of chromatographic fractions of human goblet-cell mucin*

Carbohydrate components were determined by g.l.c. of the sugar trimethylsilyl derivatives after methanolysis of mucin with 1M-HCl for 16h at 100°C.

	Carbohydrate composition (mol/100mol)	
	Mucin 1	Mucin 2
Fucose	21.7	23.7
Galactose	28.2	32.0
N-Acetylglucosamine	21.8	24.0
N-Acetylgalactosamine	23.0	19.0
N-Acetylneuraminic acid	5.3	0.8
Mannose	0	0
Hexose/protein (w/w)	1.69	5.54

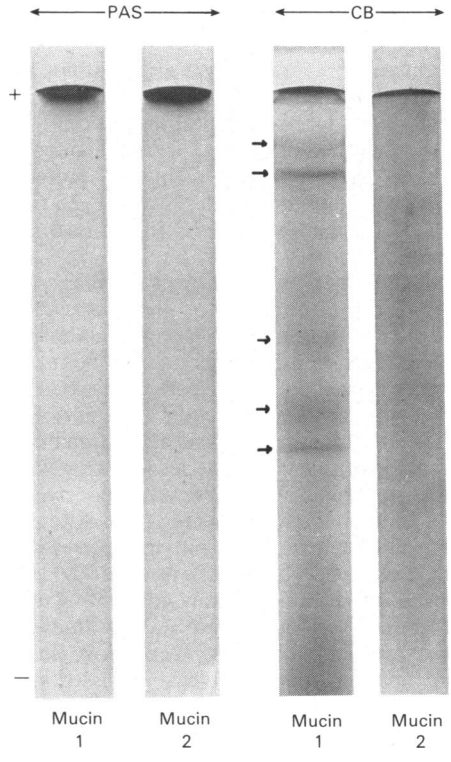

Fig. 2. *SDS/polyacrylamide-disc-gel electrophoresis of intestinal goblet-cell mucin*

Mucin preparations (mucin 1, 90 μg; mucin 2, 56 μg) were incubated for 1 h at 37°C in 1.0% SDS, and then applied to 3.5% polyacrylamide gels in 0.1% SDS and 0.15 M-Tris/borate buffer, pH 8.6. Power was applied (2 mA per tube) until the marker Bromophenol Blue dye reached the bottom of the gel. PAS indicates staining with periodic acid/Schiff reagent, and CB indicates staining with Coomassie Blue.

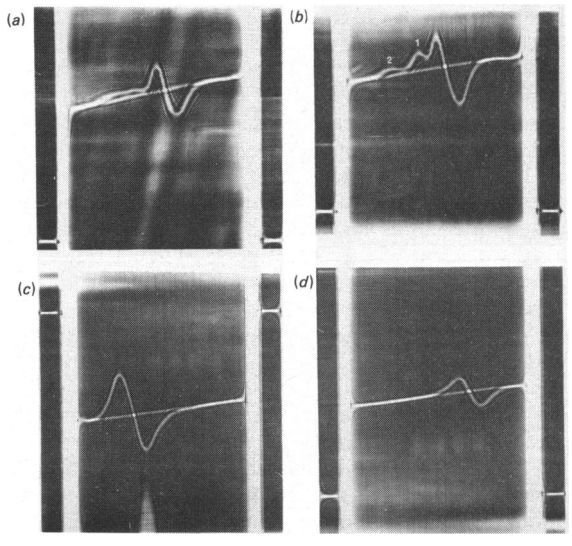

Fig. 3. *Analytical CsCl-density-gradient ultracentrifugation of mucin* 1 *before* (*a*) *and after* (*b*) *reduction and alkylation and mucin* 2 *before* (*c*) *and after* (*d*) *reduction and alkylation*

Mucin 1 samples were prepared in 43% CsCl in phosphate buffer, pH 6.8 (100 μg of protein/ml) and centrifuged for 48 h at 25°C at 44000 rev./min in a Beckman model E ultracentrifuge. Pictures were taken at an angle of 50°. The calculated buoyant-density value of the major glycoprotein species was 1.47000 g/ml in (*a*) and 1.47021 g/ml in (*b*). In the reduced sample (*b*) the densities of the minor species were calculated as 1.45513 and 1.43530 g/ml (labelled 1 and 2). In (*c*) and (*d*) the starting density of the 42.05% CsCl solution was 1.451 g/ml. Pictures were taken at an angle of 50°. The buoyant density in (*c*) was 1.47318 g/ml and in (*d*) 1.47353 g/ml.

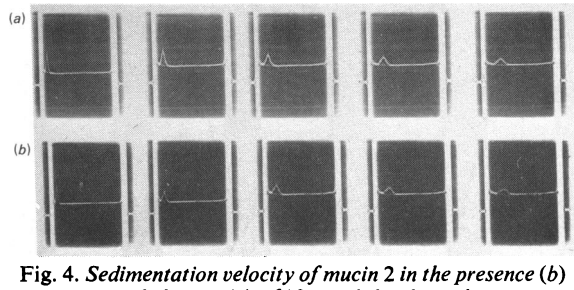

Fig. 4. *Sedimentation velocity of mucin* 2 *in the presence* (*b*) *and absence* (*a*) *of* 10 *mM-dithiothreitol*

Analyses were conducted at 20°C in a Beckman model E analytical ultracentrifuge in double-sector cells at 48000 rev./min. The solvent was 0.18 M-KCl/0.02 M-potassium acetate, pH 5.5. Mucin 2 was analysed at concentrations of 0.3, 0.8 and 1.39 mg of protein/ml. Photographs of the latter are shown for 8 min intervals (left to right). Sedimentation coefficients ($s^0_{20,w}$) were calculated from schlieren optical tracings and corrected for solvent density and viscosity. For (*a*) $s^0 = 35 \pm 2$S. For (*b*) $s^0 = 33 \pm 2$S (S.D.).

they constitute less than 0.5% by wt. of glycoprotein mixtures. Fig. 3(*a*) shows that intact mucin 1 contained a major heavy species with a buoyant density of 1.47000 g/ml. There was also some material having a slightly lower density to which accurate density values could not be assigned. There was no discrete protein contaminant, however, as judged by the tracing at the meniscus (Creeth *et al.*, 1977). The entire pattern was shown in separate experiments to be due to glycoprotein. This was done by first staining fucose and sialic acid residues of the mucin (mucin 1) with periodic acid/Schiff reagent (Jabbal *et al.*, 1975), rerunning the sample in the CsCl gradient, and monitoring the glycoprotein profile by light absorption at 555 nm. The same measured density distributions and buoyant density values were obtained (results not shown).

After reduction of mucin 1, a very different density

distribution was observed (Fig. 3*b*). The major heavy species, having a buoyant density of 1.47021 g/ml, was still present, but the lighter species became more prominent, and was resolved incompletely into two components with measured densities of 1.45513 and 1.43530 g/ml. Again, however, no low-density (< 1.4000 g/ml) peptide material was found at the meniscus. These results suggested that reduction of disulphide bonds had caused a partial degradation or dissocation of the polydisperse mucin 1 fraction. There was no evidence, however, to suggest that a cross-linking peptide had been released.

Mucin 2, the chief glycoprotein component of mucin 1, was similarly examined by analytical equilibrium ultracentrifugation in 42.05% CsCl (Fig. 3). Intact mucin 2 gave a unimodal distribution, by both schlieren optics (Fig. 3*c*) and 555 nm absorption of periodic acid/Schiff-stained samples (results not shown), which constituted good evidence for its monodispersity in terms of density. It had a measured buoyant density of 1.47318 g/ml and thus probably represents the heaviest glycoprotein species seen in mucin 1. The lighter glycoprotein species were not present in mucin 2. After reduction and alkylation of mucin 2, there was no significant change in profile or buoyant density (Fig. 3*d*).

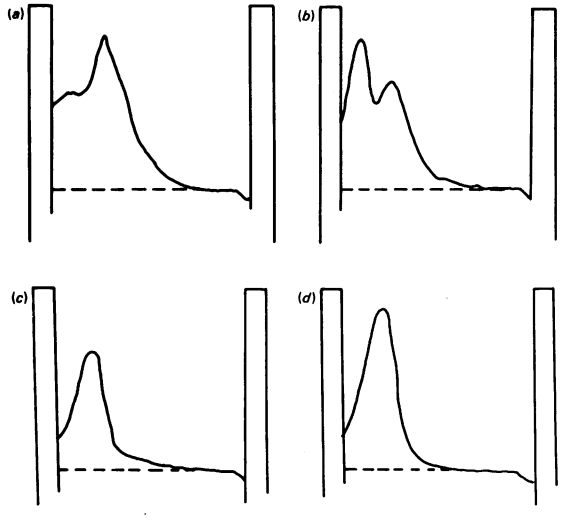

Fig. 5. *Band ultracentrifugation of mucin* 1 *before* (*a*) *and after* (*b*) *reduction and alkylation, and mucin* 2 *before* (*c*) *and after* (*d*) *reduction and alkylation, after staining with periodic acid/Schiff reagent (Jabbal et al.*, 1975)

Samples were centrifuged in a ^2H_2O-containing 0.1 M-Tris/HCl buffer, pH 7.0, at 30000 rev./min for 60 min at 20°C in a model E Beckman ultracentrifuge. Sedimentation coefficients of the major species were 37S in (*a*) and 40.8S in (*b*). The slower shoulder species (left peak in *b*) was 14.5S. The sedimentation coefficient in (*c*) was 37.3S (±2) and that in (*d*) was 33.5S (±2).

The mucin 2 preparation was also examined in the presence and absence of 10 mM-dithiothreitol by conventional boundary-sedimentation-velocity studies by using schlieren optics. Fig. 4(*b*) indicates that no changes in pattern occurred in the presence of dithiothreitol. The calculated s^0 was 35±2S (mean± S.D., $n = 3$) before, and 33±2S (mean±S.D., $n = 3$) after, reduction.

Further sedimentation analyses were carried out by band ultracentrifugation of periodic acid/Schiff-stained mucin 1 and mucin 2 samples (Jabbal *et al.*, 1975). Sedimentation coefficients could be calculated from single runs, and polydispersity assessed by the light-absorption profile at 555 nm. Figs. 5(*a*) and 5(*b*) reveal the effect of reduction and alkylation on mucin 1. The major glycoprotein species had an s^0 value of 37S (±2S.D.), and as well, there was a poorly defined slower-sedimenting shoulder (Fig. 5*a*). After reduction (Fig. 5*b*) the main faster-moving species was still present (s^0 40.8±2S), but there was a marked increase in the prominence of the slower-sedimenting material ($s^0 = 14.5$S). Therefore sedimentation studies gave results consistent with those seen above in CsCl-gradient analyses, namely a partial dissociation of mucin components as a result of thiol reduction.

When mucin 2 was subjected to band ultracentrifugation, only one major component appeared (Fig. 5*c*), having an s^0 value of 37.3S (±2S.D.). This component appears to be the same as the major species of mucin 1 seen in Fig. 5(*a*). After reduction and alkylation of mucin 2, no significant sedimentation change occurred (Fig. 5*d*), and no evidence of dissociation was observed. Therefore the major component of intestinal mucin 1 was resistant to thiol reduction, as judged by CsCl-equilibrium-density-gradient, sedimentation-velocity and band ultracentrifugation.

Discussion

Human goblet-cell mucin (1) was found to consist of two major components closely spaced in density and sedimentation velocity. The major species (mucin 2) was the denser molecule, was more neutral in charge, more highly glycosylated, and sedimented faster in the ultracentrifuge. Mucin 2 accounts for most of the isolated mucin, as judged by its carbohydrate content and density profile. The minor species appeared in small quantity from DEAE-Bio-Gel columns (peaks II and III) and was not fully characterized. It contained less carbohydrate (although more sialic acid) and more protein than did mucin 2. It was slightly less dense than mucin 2 and sedimented more slowly in the ultracentrifuge (s^0 14.5S compared with 38S). Since the lighter species separated from mucin 2 in the presence of urea during

ion-exchange chromatography, the two species are probably associated *in vivo* through non-covalent interactions.

The major glycoprotein species of the mucin (mucin 2) did not fragment into smaller or less-dense components upon treatment with disulphide-bond-breaking agents. Therefore the mucin macromolecule does not appear to be made up of smaller subunits linked together by disulphide bridges. This is distinctly different from the reported behaviour of purified pig colonic (Marshall & Allen, 1978), pig gastric (Clamp *et al.*, 1978) and human bronchial mucus glycoproteins (Roberts, 1976), and does not add support to a universal model of mucin architecture that envisages intermolecular cross-linking by disulphide bonds (Allen *et al.*, 1974; Gallagher & Corfield, 1978; Masson, 1973; Roberts, 1976; Robson *et al.*, 1975).

When the whole mucin preparation (mucin 1), which includes major and minor glycoproteins, was reduced and alkylated, there was an apparent partial degradation or disaggregation. The minor species (s^0 14.5S) underwent an incomplete separation from the major (mucin 2) species (s^0 37S), as observed from band-ultracentrifugation measurements. CsCl-density profiles confirmed this finding, showing features consistent with partial disaggregation. Thus disulphide bonds appear to play only an indirect role in holding the minor and major species together in the whole mucin. A similar but less dramatic separation of species has also been observed in mucin 1 after the addition of 6M-guanidine hydrochloride, or after 1h of heating at 37°C (I. Jabbal, G. G. Forstner & J. F. Forstner, unpublished work). These findings tend to support the notion that, *in vivo*, the minor species interact and entangle with the major species through non-covalent bonding forces. Factors that promote molecular unfolding of the minor species, such as denaturants, heat and thiol reducing agents, secondarily weaken non-covalent interactions and produce partial dissociation of the aggregates.

Consideration should therefore be given to the possibility that the minor glycoprotein species normally serve a cross-linking role in the formation of mucin aggregates. Putative cross-linking peptides in mucins have been hypothesized, as mentioned above, on the basis of studies of bronchial, gastric, cervical and ovarian mucins (Clamp *et al.*, 1978; Gallagher & Corfield, 1978; Masson, 1973; Roberts, 1976). The cross-linking agents are thought to be relatively small peptides covalently attaching large highly glycosylated mucin threads through disulphide bonds. In porcine gastric mucin, Allen *et al.* (1974) have suggested that the cross-linking peptides are in a specialized 'naked' (non-glycosylated) segment of the mucin peptide core, and are enriched in cysteine residues. On the basis of the present study, we would have to assume a different kind of cross-linking agent and a different mechanism of polymerization for human intestinal mucin. From our results it appears that cross-linking may be served by relatively poorly glycosylated mucin glycoproteins that co-purify with, and are almost indistinguishable from, larger mucin glycoproteins. The lighter cross-linking glycoproteins have a three-dimensional structure that is presumably stabilized by intramolecular disulphide bonds. That structure permits non-covalent bonding to develop between the lighter glycoproteins and the highly glycosylated (mucin 2) dense glycoproteins, which make up the bulk of the mucin. Further work to characterize the minor glycoprotein species and to explore their possible cross-linking function will be necessary to confirm this hypothesis.

This work was supported by the Medical Research Council of Canada.

References

Allen, A. & Snary, D. (1972) *Gut* **13**, 666–672
Allen, A., Pain, R. M. & Snary, D. (1974) *Faraday Discuss. Chem. Soc.* **57**, 210–220
Bhaskar, K. R. & Creeth, J. M. (1974) *Biochem. J.* **143**, 669–679
Chambers, R. E. & Clamp, J. R. (1971) *Biochem. J.* **125**, 1009–1018
Chervenka, C. H. (1970) *Manual of Methods for the Analytical Ultracentrifuge*, p. 76, Spinco Division of Beckman Instruments, Palo Alto
Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344
Clamp, J. R., Allen, A., Gibbons, R. A. & Roberts, G. P. (1978) *Br. Med. J.* **34**, 25–42
Creeth, J. M. (1978) *Br. Med. J.* **34**, 17–24
Creeth, J. M., Bhaskar, K. R. & Horton, J. (1977) *Biochem. J.* **167**, 557–569
Dunstone, J. R. & Morgan, W. T. J. (1965) *Biochim. Biophys. Acta* **101**, 300–314
Forstner, J. F., Jabbal, I. & Forstner, G. G. (1973) *Can. J. Biochem.* **51**, 1154–1166
Gallagher, J. T. & Corfield, A. P. (1978) *Trends Biochem. Sci.* **3**, 38–41
Gelman, R. A. & Vered, J. (1976) *Biochim. Biophys. Acta* **427**, 627–633
Hill, H. D., Reynolds, J. A. & Hill, R. L. (1977) *J. Biol. Chem.* **252**, 3791–3798
Holden, K. G., Yim, N., Griggs, L. J. & Weisbach, J. A. (1971) *Biochemistry* **10**, 3110–3113
Jabbal, I., Forstner, G., Forstner, J. & Kells, D. I. C. (1975) *Anal. Biochem.* **69**, 558–571
Jabbal, I., Kells, D. I. C., Forstner, G. & Forstner, J. (1976) *Can. J. Biochem.* **54**, 707–716
Jabbal, I., Kells, D. I. C., Forstner, G. G. & Forstner, J. F. (1979) *Int. Symp. Glycoconjugates 4th* in the press
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Marshall, T. & Allen, A. (1978) *Biochem. J.* **173**, 569–578
Masson, P. L. (1973) in *Cervical Mucus in Human Reproduction: a Colloquium* (Elstein, M., Moghissi, K. S. & Borth, R., eds.), pp. 82–92, Scriptor, Copenhagen

Morris, E. R. & Rees, D. A. (1978) *Br. Med. J.* **34**, 49–54
Oemrawsingh, I. & Roukema, P. A. (1974) *Arch. Oral Biol.* **19**, 753–759
Roberts, G. P. (1974) *Eur. J. Biochem.* **50**, 265–280
Roberts, G. P. (1976) *Arch. Biochem. Biophys.* **173**, 528–537
Robinson, D. S. & Monsey, J. B. (1975) *Biochem. J.* **147**, 55–62
Robson, T., Allen, A. & Pain, R. H. (1975) *Biochem. Soc. Trans.* **3**, 1105–1106
Roussel, P., Degand, P., Lamblin, G., Laine, A. & Lafitte, J. J. (1978) *Lung* **154**, 241–260
Spiro, R. G. (1966) *Methods Enzymol.* **8**, 3–25
Starkey, B. J., Snary, D. & Allen, A. (1974) *Biochem. J.* **411**, 633–639
Svennerholm, I. (1957) *Biochim. Biophys. Acta* **24**, 604–609