Biochem. J. (1981) **195**, 267–275
Printed in Great Britain

Isolation and characterization of the native glycoprotein from pig small-intestinal mucus

Michèle MANTLE and Adrian ALLEN
Department of Physiology, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received 31 October 1980/Accepted 9 December 1980*)

Glycoprotein from pig small-intestinal mucus was isolated free of non-covalently bound protein and nucleic acid with a yield of over 60%. No non-covalently bound protein could be detected by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis or by equilibrium centrifugation in a density gradient of CsCl with 4 M-guanidinium chloride. The intrinsic viscosity and reduced viscosity of the glycoprotein preparations rose with the removal of non-covalently bound protein and nucleic acid from the glycoprotein, evidence that non-covalently bound protein does not contribute to the rheological properties of the glycoprotein in the mucus. The pure glycoprotein, in contrast with impure preparations, gelled at the same concentration of glycoprotein as that present in the gel *in vivo*. The glycoprotein was a single component, as judged by gel filtration and analytical ultracentrifugation. The distribution of sedimentation coefficients was polydisperse but unimodal with an $s^0_{25,w}$ of 14.5 S and a molecular weight of 1.72×10^6. The chemical composition of the glycoprotein was 77% carbohydrate and 21% protein, 52% of which was serine, threonine and proline. The glycoprotein had a strong negative charge and contained 3.1% and 18.3% by weight ester sulphate and sialic acid respectively. The molar proportion of *N*-acetylgalactosamine was nearly twice that of any of the other sugars present, the glycoprotein had A and H blood-group activity and the average maximum length of the carbohydrate chains was deduced to be six to eight sugar residues.

In this and the following paper (Mantle *et al.*, 1981) we describe the isolation, characterization and polymeric structure of a glycoprotein that possesses the viscous and gel-forming properties of the native pig small-intestinal mucus. Preliminary studies (Mantle & Allen, 1979) have suggested that this purified glycoprotein has a relatively short carbohydrate chain and a polymeric structure that was substantially different from pig gastric mucus or pig colonic mucus glycoproteins. Therefore a comparative study between these glycoproteins would be valuable in assessing the effect of such differences in glycoprotein structure on the mucus gel, as well as providing a basis for understanding the physiology of mucus in different regions of the gastrointestinal tract.

One aspect of this work is the removal of non-covalently bound protein, which is very firmly attached to the glycoprotein and not completely removed by many of the usual separation methods, e.g. gel filtration in 0.2 M salt (Starkey *et al.*, 1974). The most successful method for removing such protein is equilibrium centrifugation in a CsCl density gradient, and this has been used preparatively for ovarian-cyst mucus (Creeth & Denborough, 1970), pig gastric mucus (Starkey *et al.*, 1974), human gastric mucus (Pearson *et al.*, 1980), pig colonic mucus (Marshall & Allen, 1978) and here for pig small-intestinal mucus glycoprotein. The presence of this protein, which is firmly but non-covalently bound to these mucus glycoproteins, has led to the suggestion that it plays an integral role in the enhancement of the rheological properties of mucus (Roberts, 1976; List *et al.*, 1978; Creeth, 1978). We show that for pig small-intestinal mucus glycoprotein the opposite occurs, namely the viscosity and gel-forming potential increase with progressive removal of the non-covalently bound protein during isolation.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental

Isolation and purification of the pig small-intestinal mucus glycoprotein

The small intestines of freshly slaughtered pigs were dissected out from the beginning of the jejunum

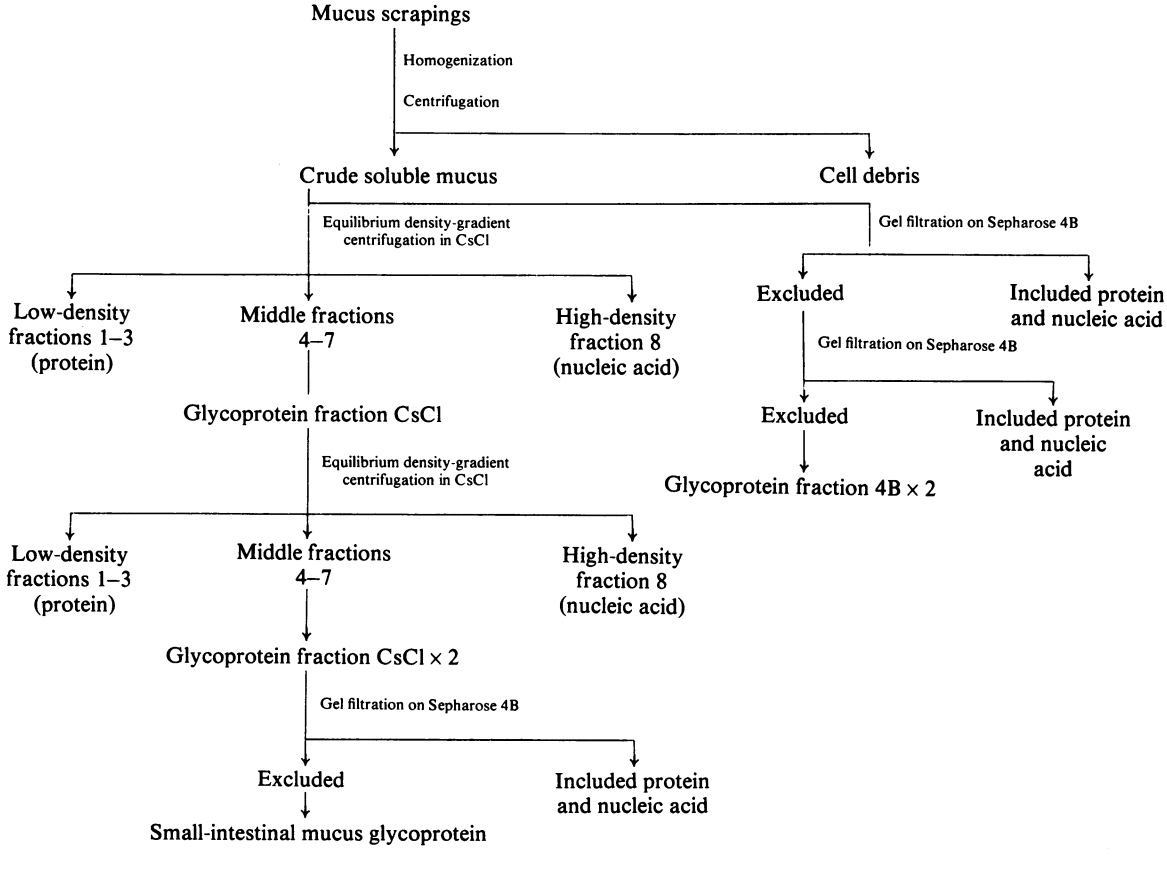
0306-3275/81/040267-09$01.50/1

to the ileo-caecal sphincter. The intestines, sectioned into short lengths, were flushed through with cold running water, and the mucosal surface was exposed by longitudinal dissection. By using a microscope slide the mucus layer was gently scraped off and collected in cold 0.2 M-NaCl containing 0.02% NaN_3. The gel was solubilized by homogenization for 30s in a Waring blender, and the soluble mucus was separated from the tissue debris by centrifugation at 6000 ***g*** for 30 min, and exhaustively dialysed against 0.04% NaN_3.

The glycoprotein was purified by the method outlined in Scheme 1. Non-covalently bound protein and nucleic acid were removed from the glycoprotein by equilibrium density-gradient centrifugation in aqueous CsCl (Creeth & Denborough, 1970; Starkey *et al.*, 1974). The glycoprotein preparation (5–12 mg/ml) was adjusted to a density of 1.43 g/ml by the addition of 0.6 g of solid CsCl per ml final volume of solution, and centrifuged at 1.5×10^5 ***g*** for 48h at 5°C. The resultant gradients were fractionated into eight equal parts and, after exhaustive dialysis, were freeze-dried before analysis.

Analytical methods

Glycoprotein was measured by the periodic acid–Schiff method (Mantle & Allen, 1978), with pig small-intestinal mucus glycoprotein as standard, nucleic acid was determined from the phosphate content (measured by the method of Gomori, 1941) and protein content was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Identification of monosaccharides by g.l.c. was kindly performed by Mr. R. A. Faulkes (National Institute for Medical Research, Mill Hill, London N.W.7, U.K.) by the method of Chambers & Clamp (1971). The protein was hydrolysed in 6 M-HCl for 24h at 105°C, with further samples hydrolysed for 48h and 72h before amino acid analysis (Starkey *et al.*, 1974). Sialic acid was liberated by hydrolysis of the purified glycoprotein in 0.05 M-H_2SO_4 for 50 min at 80°C and determined by



Scheme 1. *Preparation of pig small-intestinal mucus glycoprotein*
For experimental details see the text.

the thiobarbituric acid method of Aminoff (1961), with *N*-acetylneuraminic acid as standard. Sulphate was determined by the method of Clarke & Denborough (1971).

Enzymic digestion

The sediment obtained after homogenization and centrifugation (Scheme 1) of the mucosal scrapings was suspended in 2 vol. of 0.2 M-ammonium acetate, pH 6.5, containing 0.02% NaN_3, and digested with Pronase (EC 3.4.24.4; type VI; Sigma Chemical Co., Poole, Dorset, U.K.) for 72 h at 37°C, at an initial concentration of 0.4 mg/ml. An additional 0.2 mg of enzyme/ml was added to the digest after incubation for 24, 36 and 48 h respectively, giving a final enzyme concentration of 1 mg/ml. After exhaustive dialysis, the digest was freeze-dried and analysed for glycoprotein.

Sialic acid was removed from the purified glycoprotein (1 mg/ml in 0.02% NaN_3) by incubation with neuraminidase (EC 3.2.1.18; enzyme/substrate ratio 1:10, w/w) for 1 h at pH 6 at 37°C. The enzyme was subsequently inactivated by heating at 56°C for 30 min and the liberated sialic acid was determined by the method of Aminoff (1961). After exhaustive dialysis, the desialylated glycoprotein was freeze-dried.

Blood-group activity

Blood-group A, B and H activity was measured by the method of Kabat & Bezer (1945) over a dilution range of 1–100 μg of glycoprotein/ml in 0.85% NaCl and by using that dilution of antiserum or lectin that just gave complete agglutination of a 4% (w/v) erythrocyte suspension. The anti-(blood-group H) reagent used was the lectin from gorse (*Ulex europeus*) seeds from Sigma Chemical Co.

Ultracentrifugation studies

Ultracentrifugation studies were performed in a Beckman model E analytical ultracentrifuge in 0.18 M-KCl/0.02 M-potassium acetate/0.02% NaN_3 buffer, adjusted to pH 5.5 with acetic acid ($\eta_{rel.,25} = 1.017$; $\rho_{25} = 1.011$ g/ml).

Sedimentation-velocity analysis was performed at 60000 rev./min at 25°C, at glycoprotein concentrations of 2–10 mg/ml. Linear relations were obtained when values of $1/s_{25,w}$ were plotted against glycoprotein concentration, and $1/s^0_{25,w}$ was obtained by extrapolation to zero concentration. Diffusion coefficients of the purified glycoprotein were determined at four concentrations in the range 4–8 mg/ml, and values of $1/D_{25,w}$ when plotted against $C_0/2$ were independent of concentration and extrapolated to give $1/D^0_{25,w}$ (Creeth & Pain, 1967). Molecular weights were determined by combination of $s^0_{25,w}$ and $D^0_{25,w}$ in the Svedberg equation. The value for the partial specific volume ($\bar{v}$) used for the glycoprotein was 0.63 ml/g (Snary *et al.*, 1971). The molecular weight of the purified glycoprotein was also determined by high-speed equilibrium centrifugation, and equilibrium was attained after centrifugation at 4400 rev./min for 97 h by using the meniscus-depletion technique of Yphantis (1964). Typical glycoprotein concentrations were in the range 0.2–1.0 mg/ml. Frictional coefficients were calculated from diffusion coefficients (Creeth & Pain, 1967).

The distribution of sedimentation coefficients was calculated by the method of Sajdera & Hascall (1969). Analytical sedimentation-velocity experiments were performed at 25°C by using interference optics at a rotor speed of 30000 rev./min. Glycoprotein solutions were run at concentrations of 0.5–2 mg/ml, and the results were evaluated by a least-squares polynomial computer program (D. Mantle & R. H. Pain, unpublished work). The maximum $s_{25,w}$ from the distribution plot of sedimentation coefficients was independent of the time of centrifugation. Loss of material during ultracentrifugation was estimated by comparison of the fringe number at low (3000 rev./min) and high (30000 rev./min) speeds (Schachman, 1957).

Determination of intrinsic viscosity

Measurements were performed in a Couette rotating-cylinder viscometer at 25°C between the shear rates of 7 and 217 s^{-1} (1 and 30 rev./min). Viscosity measurements were made at glycoprotein concentrations in the range 10.0–1.0 mg/ml equilibrated with 0.18 M-KCl/0.02 M-potassium acetate/0.02% NaN_3 buffer, pH 5.5.

When the angular deflexion (in degrees) was plotted as a function of the speed of rotation of the viscometer cylinder for a number of glycoprotein concentrations, linear plots were usually obtained. In some cases shear-dependence of viscosity was present, and for such non-linear plots the slope was that of the tangent drawn at zero concentration.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was conducted by the method of Weber *et al.* (1972). The samples (5 mg/ml) were solubilized in 0.01 M-sodium phosphate buffer, pH 7.2, containing 1% (w/v) SDS and 10% (v/v) glycerol. After being heated at 100°C for 2 min, the mixture was layered on to the polyacrylamide gel [7 cm × 0.9 cm; 7.5% (w/v) polyacrylamide, 0.1% (w/v) SDS]. After electrophoresis (8 mA/gel), the gels were stained with Coomassie Brilliant Blue and destained in a 5.0% (v/v) methanol/7.5% (v/v) acetic acid mixture. The following marker proteins (0.2 mg/ml) of known molecular weight were used for standards: bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), trypsinogen (mol.wt. 24000) and lysozyme (mol.wt. 14300).

Results and discussion

Purification of small-intestinal mucus glycoprotein

Pig small-intestinal mucosal scrapings were homogenized to solubilize the mucus gel, and the glycoprotein was purified by the scheme shown in Scheme 1. The crude soluble mucus contained only about 15% by weight of glycoprotein, with protein as the major component by weight, and a small amount of nucleic acid (Table 1). Analysis of the glycoprotein-rich fraction showed that a total of 89% by weight of the protein and 27% by weight of the nucleic acid present in the crude mucus had been removed after the second CsCl density-gradient fractionation. Most of the periodic acid–Schiff-positive glycoprotein was completely excluded by the Sepharose 4B (Pharmacia, Uppsala, Sweden), used for the final purification step, but 10–15% of the material was slightly included and formed a trailing edge to the excluded peak. There was no included glycoprotein, but small included nucleic acid and protein peaks were present. The completely excluded periodic acid–Schiff-positive fractions from the column (Fig. 1) were pooled and taken as the purified small-intestinal glycoprotein. The small amount of glycoprotein in the trailing edge was not pooled with the main excluded glycoprotein peak, to avoid the inclusion of what was possibly degraded material.

The isolated intestinal mucus glycoprotein gave a single excluded peak when fractionated by gel filtration on Sepharose 2B, which excludes the presence in this preparation of any of the lower-molecular-weight proteolytically degraded glycoprotein components that are clearly included by this chromatography gel (Mantle *et al.*, 1981). The absence of nucleic acid was evident from a phosphate value of less than 0.03% by weight and the lack of ribose and deoxyribose on subsequent g.l.c. analysis. The complete removal of non-covalently bound protein was shown by (1) the absence of protein from the low-density fractions from an equilibrium centrifugation of the glycoprotein in a CsCl density gradient containing 4M-guanidinium chloride, (2) consistent amino acid analysis for seven different preparations (Table 3) and (3) no protein bands being detected when 100μl of the 5mg/ml solution of purified glycoprotein was electrophoresed on polyacrylamide gel in the presence of 0.1% SDS. Several protein bands were visible after

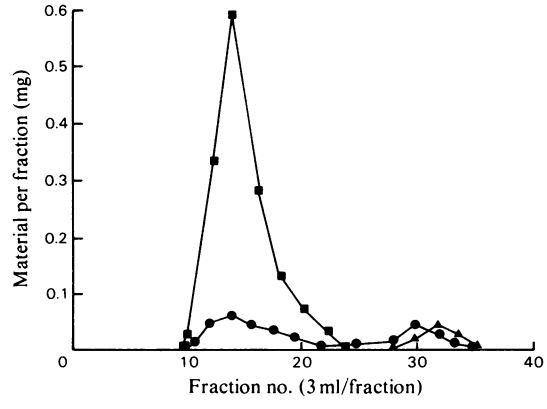


Fig. 1. *Gel filtration on Sepharose 4B of the small-intestinal mucus glycoprotein prepared by equilibrium density centrifugation (glycoprotein fraction CsCl × 2)*
Glycoprotein (5mg) was eluted from a Sepharose 4B column (40cm × 2cm). Fractions (3ml) of the eluate were analysed for protein (●), glycoprotein (■) and phosphate (▲).

Table 1. *Chemical and physical properties of the glycoprotein fractions from pig small-intestinal mucus*
For experimental details see the Experimental section. All physical data on the purified small-intestinal glycoprotein and glycoprotein fraction CsCl × 2 are averages of values from at least four different preparations, except for the equilibrium molecular weight, which is an average of three. All other data are averages of values from at least two different preparations. Protein was determined by the method of Lowry *et al.* (1951), which gives a lower value for the pure glycoprotein than that from summation of the amio acids (Table 2).

Fraction (from Scheme 1)	$s^0_{25,w}$ (S)	Intrinsic viscosity $[\eta]$ (ml/mg)	Composition (% of freeze-dried weight)		
			Glycoprotein	Protein	Nucleic acid
Crude soluble mucus	—	—	15	80	5
Glycoprotein fraction 4B × 2	13.8	0.14	71	27	2
Glycoprotein fraction CsCl	15.6	0.14	66	31	4
Glycoprotein fraction CsCl × 2	12.9	0.36	88	8.6	3.7
Small-intestinal mucus glycoprotein	14.5	0.50	93	6.8	<0.4
D^0 0.576×10^{-7} cm²/s					
Mol.wt. $(s^0 + D^0)$ 1.72 (±0.12) × 10^6					
Mol.wt. (equilibrium) 1.82 (±0.18) × 10^6					
K_s 0.185 ml/mg					
f/f_0 5.59					

SDS/polyacrylamide-gel electrophoresis of the partially purified glycoprotein preparations from the first and second fractionations in CsCl and in preparations purified by two successive fractionations on Sepharose 4B alone (fraction 4B × 2 in Scheme 1). After SDS/polyacrylamide-gel electrophoresis of all preparations there was a diffuse stain at the origin where the glycoprotein had not entered the gel.

The very high non-glycoprotein protein content of over 70% by weight and the presence of nucleic acid in the crude soluble small-intestinal mucus is comparable with preparations of pig colonic mucus (Marshall & Allen, 1978) and contrasts with similar preparations of pig gastric mucus, which contain over 60% by weight of the mucus glycoprotein (Starkey *et al.*, 1974). This is presumably due to the removal of mucosal cells during scraping of the intestinal mucosa, together with contributions from intestinal secretions and the enriched gut microflora that characterize the lower regions of the digestive tract. The three-stage procedure necessary for the isolation of pure intestinal glycoprotein emphasizes the problem of removing non-covalently bound protein, much of which is firmly attached to the glycoprotein (Creeth & Denborough, 1970; Starkey *et al.*, 1974; Creeth, 1978), and only a combination of equilibrium density-gradient centrifugation and gel filtration is satisfactory. Of the total glyco-substance in mucosal scrapings 19% by weight remained in the precipitate after centrifugation of the homogenate; therefore over 80% of the mucus glycoprotein present was solubilized, probably more, since membrane glycoproteins and glycolipids will account for some of the periodic acid–Schiff-positive material in the insoluble precipitate. Of the soluble glycoprotein preparation 71% by weight was recovered as purified small-intestinal glycoprotein, and this value too is an underestimation since it does not include the trailing peak from the final stage of purification of the glycoprotein on Sepharose 4B (Fig. 1) or the glycoprotein lost by discarding fraction 8 from the two equilibrium density-gradient centrifugations (Scheme 1). Thus the isolated purified glycoprotein represented 60% or more by weight of the glyco-substance in the original small-intestinal mucus scrapings. Therefore this glycoprotein must represent the principal, if not the only, glycoprotein component of the small-intestinal mucus gel.

Physical analysis of the glycoprotein preparations

Sedimentation-velocity analysis of the purified intestinal glycoprotein produced a single symmetrical peak, hypersharp at high concentrations and polydisperse at low. Sedimentation coefficients were concentration-dependent, and an $s^0_{25,w}$ of 14.5S (±1.14S) was obtained with a K_s of 185ml/g, as an average for four independent glycoprotein preparations (Table 1). Counts of the interference-fringe patterns at low speed and high speed gave 12.9 fringes at 3000rev./min and 12.7 fringes at 30000rev./min for a 5mg/ml solution, indicating no loss of glycoprotein during centrifugation.

The distribution of $s_{25,w}$ values, $g(S)$, was determined by using interference optics by the method of Sajdera & Hascall (1969). The distribution plot was symmetrical (Fig. 2), and the peak maximum gave an $s_{25,w}$ of 13.6S for a 0.7mg/ml solution of the glycoprotein, which was found to lie on the plot of $1/s_{25,w}$ against glycoprotein concentration. The distribution plot of sedimentation coefficients was broad but symmetrical, with no evidence for the presence of separate peaks. This indicates that the small-intestinal mucus glycoprotein is polydisperse with respect to size, like other mucus glycoproteins, but that the unimodal nature of its size distribution is evidence that it is a single component (Gibbons *et al.*, 1970; Gibbons, 1972).

Static diffusion coefficients were determined at four concentrations, and were found to be independent of concentration. When $D^0_{25,w}$ and $s^0_{25,w}$ were combined in the Svedberg equation, a value of $1.72(\pm 0.1) \times 10^6$ was obtained for the molecular weight of the purified glycoprotein. The molecular weight of the pure small-intestinal glycoprotein was also determined from high-speed equilibrium centrifugation by the method of Yphantis (1964), and a value of $1.82(\pm 0.17) \times 10^6$ was obtained. The close agreement between the two different molecular-weight determinations for the small-intestinal glycoprotein is better than that found for some other purified preparations, including ovarian-cyst glyco-

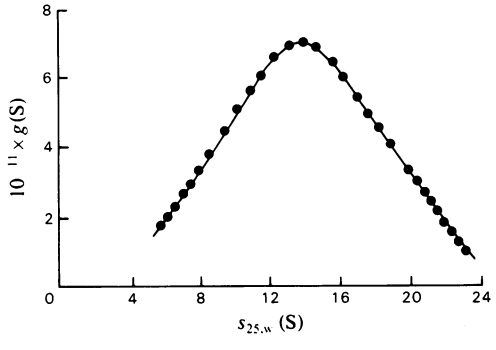


Fig. 2. *Distribution of sedimentation coefficients for the purified glycoprotein*

Glycoprotein (0.7mg/ml) in 0.2M-KCl/0.02M-potassium acetate/0.02% NaN_3 buffer, pH5.5, was centrifuged at 30000rev./min in a Beckman model E analytical centrifuge. The photograph from which the distribution was determined was taken 49s from the start of centrifugation.

protein (Gibbons *et al.*, 1970) and pig gastric glycoprotein (Starkey *et al.*, 1974), and again emphasizes the homogeneity of this glycoprotein.

The intrinsic viscosity of the purified small-intestinal glycoprotein was high (500ml/g), with the reduced specific viscosity rising asymptotically as the concentration of glycoprotein increased until about 10–12mg/ml, when it formed a gel (Fig. 3). Very small quantities of intact native gel could be obtained directly from the small-intestinal mucosal surface and apparently free of mucosal tissue. These had a glycoprotein content of 10.5 ± 0.5mg/ml from two separate estimations. Although this value is approximate, it does show that the isolated purified glycoprotein can form a gel in the same concentration range as that found for the mucus gel *in vivo*. Since gel formed by the isolated small-intestinal glycoprotein is soluble in 0.2M-NaCl, it must therefore be formed by non-covalent interactions between the glycoprotein molecules. Calculations from the sedimentation data of the effective hydrodynamic volume (V_e) for the small-intestinal mucus glycoprotein (Yang, 1961) gave a value of 111ml/g. V_e is a measure of the volume within which the probabilty of molecular interactions becomes appreciable, and calculation shows that for the small-intestinal glycoprotein this would be about 9mg/ml when the glycoprotein occupies the whole of the solution volume. The viscosity data, presented in Fig. 3, support this conclusion, since the steep rise in viscosity, characteristic of intermolecular gel-forming interactions, becomes appreciable at about a glycoprotein concentration of 9mg/ml. From these data the mechanism of gel formation by small-intestinal mucus glycoprotein would appear to be the same as that postulated for pig gastric mucus glycoprotein (Allen *et al.*, 1976), where the expanded hydrated glycoprotein molecules occupy the total solution volume, and where they overlap non-covalent gel-forming interactions occur. The concentration of glycoprotein in the pig small-intestinal mucus gel (10mg/ml) is rather lower than that for pig gastric mucus (about 50mg/ml) and implies that the former has a more open molecular matrix.

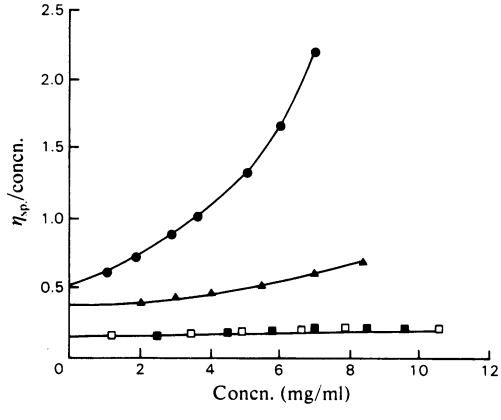


Fig. 3. *Viscosity of preparations of pig small-intestinal mucus glycoprotein*

All solutions were measured in 0.2M-KCl/0.02M-potassium acetate/0.02% NaN_3 buffer, pH5.5, in a Couette viscometer over the shear range 1–30rev./min. ●, Pure glycoprotein; ▲, glycoprotein fraction CsCl × 2; ■, glycoprotein fraction CsCl × 1; □, glycoprotein fraction 4B × 2.

The intrinsic viscosities of the different glycoprotein fractions obtained during purification increased as the free protein and nucleic acid were removed from the glycoprotein preparation (Table 1). Further, as the purity of the preparation increased there was an increase in the slope of the curve (Fig. 3) when the reduced specific viscosity was plotted against total concentration. The intrinsic viscosity of the glycoprotein fraction CsCl × 2 (Fig. 3 and Table 1) is what would be expected if the concentration of undegraded glycoprotein present in this material alone determined the viscosity. For the more impure preparations, glycoprotein fractions CsCl and 4B × 2, the intrinsic viscosity and specific viscosity at given concentrations are appreciably lower than those expected for the amount of undegraded glycoprotein present. These results show that the glycoprotein alone determines the gel-forming properties of the secreted mucus, and contrast with the proposal that non-covalent interactions between glycoprotein and protein can enhance the viscosity and gel-forming properties of these molecules (Creeth, 1978; List *et al.*, 1978). Our results here also emphasize the importance of obtaining the glycoprotein entirely free of non-covalently bound protein and nucleic acid if a true assessment of its rheological properties is to be obtained. It would seem reasonable to suggest that *in vivo* small-intestinal mucus gel is secreted as relatively pure glycoprotein, and that contamination with protein and nucleic acid occurs when mucus is scraped from the mucosal surface and homogenized during extraction of the glycoprotein.

The $s^0_{25,w}$ value is unchanged for the glycoprotein preparations whether prepared by gel filtration alone or by equilibrium centrifugation in CsCl (Scheme 1 and Table 1). Thus neither the high salt concentration of CsCl (3.5M) nor the removal of non-covalently bound protein from the small-intestinal glycoprotein results in conformational changes that affect its sedimentation coefficient. This differs from pig gastric mucus glycoprotein, where there is a change in $s^0_{25,w}$ from 20S to 33S after equilibrium centrifugation in a CsCl density gradient (Snary *et al.*, 1974).

Chemical analysis of the purified small-intestinal glycoprotein

The glycoprotein consists of 77% by weight carbohydrate, 3.1% by weight ester sulphate and 21.4% by weight protein, after correction for a moisture content of 16% by weight (Tables 2 and 3), and it contains a spectrum of residues of monosaccharides and amino acids characteristic of other mucus-type glycoproteins (Gottschalk, 1972; Horowitz, 1977). Traces of mannose were also present in the purified glycoprotein, something previously noted for other purified gastrointestinal mucus glycoprotein (Carlson, 1968; Scawen & Allen, 1977). The glycoprotein contained a large number of negatively charged groups as ester sulphate and sialic acid. For sialic acid the value by colorimetric estimation was 13.0% by weight compared with a value of 18.25% by weight from g.l.c. analysis. This could be due to the presence of sialic acid residues containing *N*-glycollyl and *O*-acetyl groups, which have a lower absorbance than *N*-acetylneuraminic acid in the thiobarbituric acid assay method (Aminoff, 1961).

The glycoprotein had blood-group-substance A and H activity of 0.6 μg/0.1 ml, which is comparable with that found for purified pig gastric glycoprotein (Snary & Allen, 1971) and higher than that for pig colonic mucus (Marshall & Allen, 1978). After acid hydrolysis, which removed all of the bound sialic acid, or neuraminidase digestion, which removed 73% of the bound sialic acid, the blood-group-substance activity of the glycoprotein was 0.4 μg/0.1 ml and 0.6 μg/0.1 ml respectively. Since removal of the sialic acid does not affect A and H blood-group-substance activity, it is unlikely that the sugar residues responsible for the antigenic activity are the site of attachment of most of the sialic acid residues. No blood-group B activity was present in the glycoprotein.

The molar proportions of the individual sugars are particularly interesting, since that of galactosamine is nearly twice that of any of the other sugar residues (Table 2). In mucus glycoproteins, *N*-acetylgalactosamine has only been found to occur at either end of the sugar chains; it is the sugar that links the carbohydrate side chain to the protein core by *O*-seryl or *O*-threonyl linkages (Carlson, 1977), and sometimes it is also present at the non-reducing end of the chain, e.g. in A blood-group-substance (Watkins, 1966). If it is assumed that in pig small-intestinal mucus glycoprotein the *N*-acetylgalactosamine residues are only at the ends of the sugar chains, then the average number of sugar residues per chain would be a maximum of six to eight, depending on whether there were one or two *N*-acetylgalactosamine residues at the non-reducing ends of the sugar chains respectively. This assumes *N*-acetylgalactosamine residues at the non-reducing ends of every chain, but the average length of the chains would be less if some are incomplete without such a residue at the non-reducing end of the chains. The strong A and H blood-group activity of the

Table 2. *Chemical composition of pig small-intestinal mucus glycoprotein*

The monosaccharides were determined by g.l.c. and the protein content was calculated by summation of the individual amino acid contents (Table 3). The results are averages of determinations on seven independent glycoprotein preparations. The moisture content was determined by drying to constant weight.

	Composition (% by wt. of freeze-dried glycoprotein)	Molar proportions of sugar residues
Fucose	4.38	0.26
Mannose	0.81	0.04
Galactose	12.11	0.64
Galactosamine	18.89	1.0
Glucosamine	10.35	0.55
Sialic acid	18.25	0.56
Sulphate	2.6	0.31
Protein	17.95	—
Moisture	16.0	—

Table 3. *Amino acid composition of the small-intestinal mucus glycoprotein*

The glycoprotein was analysed by the method of Spackman *et al.* (1958); the cysteine content was determined by prior reduction and carboxymethylation of the purified glycoprotein. The data are averages of seven determinations (three for cysteine) on independent glycoprotein preparations.

Amino acid	Amino acid composition (nmol/mg dry wt. of glycoprotein)	Amino acid composition (mol/100 mol of the total protein)
Lys	39 ± 7	2.22
His	24 ± 2	1.37
Arg	39 ± 4	2.22
Asp	81 ± 10	4.63
Thr	464 ± 35	26.53
Ser	182 ± 10	10.40
Glu	74 ± 7	4.20
Pro	270 ± 13	15.44
Gly	98 ± 4	5.60
Ala	66 ± 6	3.77
½Cys	75 ± 2	4.28
Val	125 ± 11	7.15
Met	22 ± 4	1.25
Ile	55 ± 6	3.14
Leu	75 ± 6	4.28
Tyr	26 ± 9	1.46
Phe	35 ± 9	2.00

glycoprotein indicates that galactose and fucose must be situated at the non-reducing ends of the sugar chains (Watkins, 1966). For the short chains proposed above, this would mean that the inner residues would be *N*-acetylglucosamine and the *N*-acetylgalactosamine joined to the serine and threonine. The sialic acid residue must be terminal, since most of it is removed by neuraminidase, but its removal does not affect blood-group activity, and therefore it is more likely to be attached to the inner *N*-acetylglucosamine or *N*-acetylgalactosamine. The molar proportions of fucose and ester sulphate in relation to the other sugars suggest that only some of the carbohydrate chains carry these residues. The exact structure of the carbohydrate side chains of small-intestinal mucus glycoprotein must await detailed chemical analysis.

Serine, threonine and proline comprise 52.4% by weight of the protein core of small-intestinal glycoprotein (Table 3), and this high content is characteristic of these mucus glycoproteins, although mucus glycoproteins isolated by proteolytic digestion have still higher contents of these three amino acids, over 70% by weight of the protein (Allen, 1978). The amino acids content is similar to that found in native pig gastric and colonic mucus glycoproteins, although the molar proportions of individual amino acids are different. The small-intestinal glycoprotein contains significant amounts of half-cystine residues (4.3% by weight) and is low in its content of aromatic residues (Table 3).

There are clearly marked differences between the carbohydrate chains of the glycoproteins from the different regions of the pig gastrointestinal tract. Pig submaxillary-gland mucus glycoprotein, with five sugars (Carlson, 1968), has the simplest structure, and from the above pig small-intestinal mucus glycoprotein carbohydrate chains are apparently not much larger in size, although they contain *N*-acetylglucosamine residues. In contrast with these glycoproteins, the carbohydrate side chain of pig gastric mucus glycoprotein is much more complex, with a maximum chain length of 19 sugar residues (Slomiany & Meyer, 1972). The structure of the carbohydrate side chains of pig colonic mucus is unknown, but there is only a low molar proportion of *N*-acetylgalactosamine to the other sugars, and the analysis is close to that of pig gastric glycoprotein (Marshall & Allen, 1978). Despite these differences in sugar composition and size of carbohydrate side chains, all these pig gastric intestinal mucus glycoproteins possess strong A and H blood-group antigenic activity.

A further difference between the mucus glycoproteins from the various regions of the pig gastrointestinal tract is the amount of negative charge on the carbohydrate side chains. Pig submaxillary-gland mucus glycoprotein has about one sialic acid residue per carbohydrate chain (Carlson, 1968), and pig gastric mucus glycoprotein has an ester sulphate content that is equivalent to about one per 15 sugar residues (Slomiany & Meyer, 1972; Scawen & Allen, 1977) together with a small amount of sialic acid (Allen & Starkey, 1974). Pig small-intestinal mucus glycoprotein, however, is rich in both sialic acid and ester sulphate residues (Table 2). Pig colonic mucus also has a high content of both sialic acid and ester sulphate residues (Marshall & Allen, 1978). It should be pointed out that, despite these differences in carbohydrate structure and negative charge, pig gastric and pig intestinal glycoproteins both form typical mucus gels.

Pig intestinal mucus glycoprotein has a molecular weight of 1.7×10^6, which is similar in size to the other small-intestinal mucus glycoproteins that have been isolated, by procedures not involving equilibrium centrifugation in CsCl, from rat small intestine (Bella & Kim, 1972; Forstner *et al.*, 1973) and human small intestine (Jabbal *et al.*, 1976; Forstner *et al.*, 1979). However, there are differences in the molar proportions of the constituent sugar residues; in particular, both rat and human intestinal glycoproteins have a low *N*-acetylgalactosamine content relative to the other sugars, although the human preparations were from non-secretors, where *N*-acetylgalactosamine would be missing from the non-reducing ends of the chain. It is noteworthy that the sulphated glycopeptide antigen from human gastric tumours, which is localized at sites of intestinal metaplasia and in the goblet cells, has monosaccharide molar proportions close to those of pig small-intestinal mucus, and in particular contains more *N*-acetylgalactosamine than any of the other sugars (Bara *et al.*, 1978).

We thank Mr. R. Faulkes for doing the g.l.c. analysis, Mr. R. Adams for his help, and the Medical Research Council for financial support.

References

Allen, A. (1978) *Br. Med. Bull.* **34**, 28–33

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

Allen, A., Pain, R. H. & Robson, T. (1976) *Nature (London)* **264**, 88–89

Aminoff, D. (1961) *Biochem. J.* **81**, 384–392

Bara, J., Paul-Gardais, A., Loisillier, F. & Burtin, P. (1978) *Int. J. Cancer* **21**, 133–139

Bella, A. & Kim, Y. S. (1972) *Arch. Biochem. Biophys.* **150**, 679–689

Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626

Carlson, D. M. (1977) in *Mucus in Health and Disease* (Elstein, M. & Parke, D. V., eds.), pp. 251–273, Plenum Press, New York and London

Chambers, R. E. & Clamp, J. R. (1971) *Biochem. J.* **125**, 1009–1018

Clarke, A. E. & Denborough, M. A. (1971) *Biochem. J.* **121**, 811–816
Creeth, J. M. (1978) *Br. Med. Bull.* **34**, 17–24
Creeth, J. M. & Denborough, M. A. (1970) *Biochem. J.* **117**, 879–891
Creeth, J. M. & Pain, R. H. (1967) *Prog. Biophys. Mol. Biol.* **17**, 217–287
Forstner, J. F., Jabbal, I. & Forstner, G. G. (1973) *Can. J. Biochem.* **51**, 1154–1166
Forstner, J. F., Jabbal, I., Qureshi, R., Kells, D. I. C. & Forstner, G. G. (1979) *Biochem. J.* **181**, 725–732
Gibbons, R. A. (1972) in *Glycoproteins* (Gottschalk, A., ed.), pp. 31–109, Elsevier, Amsterdam and New York
Gibbons, R. A., Creeth, J. M. & Denborough, M. A. (1970) in *Blood and Tissue Antigens* (Aminoff, D., ed.), p. 307–323, Academic Press, New York
Gomori, G. (1941) *J. Lab. Clin. Med.* **27**, 955–960
Gottschalk, A. (ed.), (1972) *Glycoproteins*, Elsevier, Amsterdam and New York
Horowitz, M. I. (1977) in *The Glyconjugates* (Horowitz, M. I. & Pigman, W., eds.), pp. 189–213, Academic Press, New York
Jabbal, I., Kells, D. I. C., Forstner, G. G. & Forstner, J. F. (1976) *Can. J. Biochem.* **54**, 707–716
Kabat, E. A. & Bezer, A. E. (1945) *J. Exp. Med.* **82**, 207–215
List, S. J., Findlay, B. P., Forstner, G. G. & Forstner, J. F. (1978) *Biochem. J.* **175**, 565–571
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mantle, M. & Allen, A. (1978) *Biochem. Soc. Trans.* **6**, 607–609
Mantle, M. & Allen, A. (1979) *Biochem. Soc. Trans.* **7**, 393–395
Mantle, M., Mantle, D. & Allen, A. (1981) *Biochem. J.* **195**, 277–285
Marshall, T. & Allen, A. (1978) *Biochem. J.* **173**, 569–578
Pearson, J. P., Allen, A. & Venables, C. W. (1980) *Gastroenterology* **78**, 709–715
Roberts, G. R. (1976) *Arch. Biochem. Biophys.* **173**, 528–537
Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
Scawen, M. & Allen, A. (1977) *Biochem. J.* **163**, 363–368
Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–103
Slomiany, B. L. & Meyer, K. (1972) *J. Biol. Chem.* **247**, 5062–5070
Snary, D. & Allen, A. (1971) *Biochem. J.* **123**, 845–853
Snary, D., Allen, A. & Pain, R. H. (1971) *Eur. J. Biochem.* **24**, 183–189
Snary, D., Allen, A. & Pain, R. H. (1974) *Biochem. J.* **141**, 641–646
Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
Starkey, B. J., Snary, D. & Allen, A. (1974) *Biochem. J.* **141**, 633–639
Watkins, W. M. (1966) *Science* **152**, 172–181
Weber, K., Pringle, J. R. & Osborn, M. (1972) *Methods Enzymol.* **26**, 3–27
Yang, J. T. (1961) *Adv. Protein Chem.* **16**, 323–400
Yphantis, D. A. (1964) *Biochemistry* **3**, 297–317