Purification and characterization of goblet-cell mucin of high M_r from the small intestine of sheep

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Crude soluble mucus from sheep small intestine was freed of nearly all the nucleic acid contaminants by precipitation with protamine sulphate and treatment with nucleases. After removal of non-covalently bound proteins by equilibrium density-gradient centrifugation in CsCl, a high- M_r glycoprotein was isolated by repeated h.p.l.c. from the partially purified mucin. The high degree of purity of the high- M_r mucin was borne out by (a) the observation of a single boundary on analytical ultracentrifugation in the presence of 5 M-guanidinium chloride and (b) the observation of apparent monodispersity on sedimentation-equilibrium analysis. The M_r of the highly purified mucin, determined by sedimentation equilibrium, was $5.0 (\pm 0.1) \times 10^6$ and was concentration-independent. Finally, only goblet cells and the mucus blanket lining the intestinal epithelial cells were immunofluorescent when guinea-pig anti-(highly purified mucin) serum was used in an indirect immunofluorescence assay. The above antiserum reacted with apparently equal strength with goblet cells and with free mucin in abomasum, caecum and colon. The chemical composition of the glycoprotein was 66% carbohydrate and 34% protein, 45% of the latter being composed of valine and threonine. The glycoprotein migrated anodally on immunoelectrophoresis and contained 7.1% (w/w) sulphate. Neutral hexoses accounted for nearly half of the total carbohydrate content, followed by galactosamine and glucosamine. Whereas fucose and sialic acid were present in only small amounts, uronic acid was not detectable in the highly purified mucus glycoprotein.

Intestinal mucus is a viscous glycoprotein (referred to below as mucin) overlying the epithelial surface and is produced by goblet cells (Trier, 1968; Forstner *et al.*, 1973a). Apart from the clear function of protecting the mucosal epithelium from mechanical damage (Allen, 1981), it has been suggested that mucus may be involved in gastrointestinal defence against disease, as in the immune expulsion of intestinal nematode infections including *Nippostrongylus brasiliensis* and *Trichinella spiralis* (Lee & Ogilvie, 1981; Miller *et al.*, 1981) and in preventing the adhesion of *Vibrio cholerae* to the rabbit intestinal mucosa, possibly by acting in concert with specific antibody (Schrank & Verwey, 1976).

Abbreviation used: SDS, sodium dodecyl sulphate.

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Much information is now available on isolation methods and structural properties of gastrointestinal mucus of pigs (Snary & Allen, 1971; Starkey *et al.*, 1974; Marshall & Allen, 1978; Mantle & Allen, 1981; Mantle *et al.*, 1981), rats (Forstner *et al.*, 1973b, 1979; Fahim *et al.*, 1983) and humans (Pearson *et al.*, 1980; Younan *et al.*, 1982). In sheep, the animal model that we use in our studies on mucosal defence mechanisms, although the isolation and characterization of submaxillary mucin has been reported (Hill *et al.*, 1977), little is known about gastrointestinal mucin (Kent & Marsden, 1963; Kent & Allen, 1967). In the present paper we report a method for obtaining highly purified mucin of high M_r from sheep intestine. The mucin thus isolated was physicochemically and immunochemically characterized.

Experimental

Purification of the sheep small-intestinal mucus glycoprotein

The method used to obtain partially purified mucin was essentially that of Mantle & Allen (1981). Briefly, the small intestine was cut into 10–15 cm segments, flushed through with cold saline (0.9% NaCl) followed by running water, and the mucosal surface exposed by incision along the anti-mesenteric border. The mucus layer was gently scraped off, collected in 3.0M-KCl/50mM-Tris/HCl buffer, pH 7.5, containing 1mM-phenylmethanesulphonyl fluoride, 0.1% soya-bean trypsin inhibitor and 0.05% NaN_3 , and homogenized in a Waring blender for about 1 min. The soluble mucus was separated from the tissue debris by centrifugation at 27500g for 30 min, followed by exhaustive dialysis against 0.32M-NaCl/10mM-Tris/HCl buffer, pH 8.0 (0.32 ST). Protamine sulphate was added to the crude soluble mucus to a final concentration of 10mg/ml, and the mixture was left at 4°C for 1 h before centrifugation at 27500g for 30 min to remove the precipitated nucleic acid contaminants. This was followed by further additions of protamine sulphate and centrifugations until no further precipitation was noticed (see the text below). The glycoprotein was then dialysed against 0.2M-sodium acetate buffer, pH 5.0, for digestion with deoxyribonuclease, or the same buffer at pH 7.0 for digestion with ribonuclease, and the reactions were carried out as described previously (Anon, 1972). After these enzyme digestions, the crude soluble mucus was dialysed exhaustively against 0.32 ST, and the mucus glycoprotein was obtained in an enriched form essentially as described by Mantle & Allen (1981), as summarized in Scheme 1. The non-covalently bound proteins were removed from the glycoprotein by equilibrium density-gradient centrifugation in aqueous CsCl by adjusting the density of the glycoprotein preparation to 1.43 g/ml and then centrifuging at 105000g for 60 h at 4°C. The resulting gradients were fractionated into eight equal parts and dialysed exhaustively against deionized distilled water. The higher-density fractions (5–7) were pooled (glycoprotein CsCl I) and subjected to further CsCl-density-gradient centrifugation as described above. The higher-density fractions (6–8) thus obtained were pooled (glycoprotein CsCl II or partially purified mucin), dialysed against 0.2M-ammonium acetate buffer, pH 6.0, concentrated by pervaporation, and then subjected to gel-permeation h.p.l.c. on an LKB Blue column (Ultrapac TSK-G4000SW; 7.5 mm $\times$ 600 mm). The leading peak was rechromatographed on the same column to free it from the trailing contaminants, and the remainder was

concentrated by pervaporation. After dialysis against 10mM-sodium phosphate buffer, pH 7.2, the preparation was stored frozen at -20°C .

Analytical methods

Total carbohydrate content was measured by the periodic acid/Schiff method (Mantle & Allen, 1978), with glycogen as a standard, and total protein content was determined by the method of Hartree (1972). The method of Ceriotti (1952) was used for measurement of the DNA content, and RNA was measured as described by Johnson (1981). Fucose was determined by the cysteine/ H_2SO_4 reaction (Dische & Shettles, 1948). Sialic acid was liberated by very mild acid hydrolysis of purified mucin in 50mM- H_2SO_4 at 80°C for 1 h and determined by the thiobarbituric acid assay (Aminoff, 1961), with *N*-acetylneuraminic acid as standard. Total neutral hexoses were determined by the anthrone reaction (Mokrasch, 1954). The uronic acid assay method was that of Bitter & Muir (1962). Sulphate was determined by the method of Dodgson (1961), and P_i by that of Baginski *et al.* (1974) with minor modifications.

Enzyme assays

Various marker enzyme activities assayed included lactate dehydrogenase as a cytoplasmic marker (Bergmeyer & Bernt, 1974), alkaline phosphatase as a plasma-membrane marker (Walter & Schutt, 1974), glutamate dehydrogenase as a mitochondrial marker (Schmidt, 1974), glucose-6-phosphatase as an endoplasmic-reticulum marker (Baginski *et al.*, 1974) and β -*N*-acetylglucosaminidase as a lysosomal marker (Sellinger *et al.*, 1960).

SDS/polyacrylamide-gel electrophoresis

The SDS/polyacrylamide-gel electrophoresis of crude soluble mucus and of partially purified and highly purified mucin preparations was carried out in 3.3%-polyacrylamide gels as described by Weber & Osborn (1969), but in the absence of 2-mercaptoethanol. All gels were stained with Coomassie Blue, Kenacid R and periodate/Schiff reagent (Zacharius *et al.*, 1969), and also by the silver staining method (Merril *et al.*, 1981). The M_r markers used were in the range 56000–280000 (BDH Chemicals, Poole, Dorset, U.K.).

Analytical ultracentrifugation

A Beckman model E analytical ultracentrifuge with a mechanical speed control and u.v. optics was used for sedimentation equilibrium, whereas schlieren optics were used for sedimentation-velocity runs. Whereas the sedimentation velocity runs in the acetate buffer were made in an An-D rotor at 37020 rev./min, those in guanidinium chloride were made at 42010 rev./min. Correction

Scheme 1. Protocol for the purification of high- M_r mucin from sheep small intestine
For experimental details see the text.

of observed s values to $s_{20,w}$ was effected by the method of Schachman (1957). Sedimentation-equilibrium runs were made at 4908 rev./min at 25°C in an An-J rotor by using interference optics, and the M_r values were determined for different concentrations of purified mucin by the meniscus-depletion method of Yphantis (1964) from data collected all the way to the cell bottom. Partial specific volume ($\bar{v}$) was determined employing the $H_2O/{}^2H_2O$ method of Edelstein & Schachman (1967).

Immunological tests

The immunological purity of the isolated mucin was tested by immunoelectrophoresis (Scheidegger, 1955) and the Ouchterlony (1949) gel-diffusion technique.

Preparation of antisera

Guinea pigs were inoculated in the foot-pads with 200 µg of crude soluble mucus or highly purified mucin preparations emulsified with an equal volume of aluminium hydroxide adjuvant

followed by immunizations by the intramuscular route at biweekly intervals for 2 months. Blood was then collected from the anaesthetized guinea pigs by cardiac puncture, and the sera obtained were stored at -20°C. Other antisera employed were rabbit anti-(normal sheep serum), anti-IgG, anti-IgM and anti-IgA, and were prepared as described previously (Lascelles & McDowell, 1970; Watson *et al.*, 1972).

Histology and immunohistology

Tissues were collected from various parts of the intestinal tract (abomasum, duodenum, jejunum, ileum, caecum, colon) of freshly slaughtered sheep. They were immediately placed in ice-cold 95% (v/v) ethanol and processed by the method of Sainte-Marie (1962).

For identification of glycoprotein the sections were stained with periodate/Schiff reagent (Glegg *et al.*, 1952). Immunofluorescent examinations were made as previously described (Bennell & Watson, 1979), by using incident-light fluores-

cence. Briefly, the sections were soaked for 2 h with guinea-pig anti-(highly purified mucin) serum at optimal dilution. After three washes with phosphate-buffered saline (Bennell & Watson, 1979), the sections were stained for 2 h with goat anti-(guinea-pig IgG)-fluorescein isothiocyanate conjugate (Cappel Laboratories, Malvern, PA, U.S.A.) at a dilution of 1:20, then washed and mounted.

Results and discussion

Purification of the high- M_r -glycoprotein from sheep small-intestinal mucus

The procedure developed for purification of sheep small-intestinal mucin and presented in the protocol (Scheme 1) differs from procedures previously described for purification of pig and human mucins in that (a) protamine sulphate was used to precipitate out much of the nucleic acid contaminants from the crude soluble mucus and (b) h.p.l.c. was applied as a final purification step in the preparation of high- M_r mucin. Treatment of crude soluble mucus with protamine sulphate resulted in the precipitation of 75–80% of the nucleic acids (Table 1). This was accompanied by a loss of about 10% of protein also. On digestion with deoxyribonuclease and ribonuclease, whereas no DNA was detectable by the method used in this investigation (detection limit 2.5 µg/ml), approx. 3% of the original RNA content was still detectable spectrophotometrically, although no P_i could be detected.

The crude soluble mucus was subjected to CsCl-density-gradient centrifugation (Mantle & Allen, 1981). The higher-density fractions 5–7 (CsCl I) were pooled (Fig. 1) and subjected to further CsCl-density-gradient centrifugation. Again, the higher-density fractions 6–8 (CsCl II) with high carbohydrate/protein ratio were pooled (Fig. 2), concentrated by pervaporation, dialysed exhaustively against 0.2M-ammonium acetate buffer, pH 6.0 (referred to below as 'partially purified mucin'), and subjected to h.p.l.c. on the LKB Blue column. At least four peaks (I–IV) were obtained (Fig. 3a),

all of which were eluted within the void volume (V_0) of the column, the latter having been determined by using the high- M_r giant-keyhole-limpet haemocyanin. The leading edge of peak I was concentrated and subjected to further fractionation by h.p.l.c., which gave rise to a single symmetrical peak on rechromatography (Fig. 3b). The mucus glycoprotein thus obtained was termed highly purified mucin.

As with pig colonic mucus (Marshall & Allen, 1978), the CsCl-density-gradient centrifugation was important for dissociation of the non-mucin contamination from the sheep small-intestinal mucin. All the marker enzymes assayed as indicators for the presence of various cellular organelles revealed that the latter were present in the top of the CsCl density gradient (Table 2). The higher-density fractions, representing partially purified mucin, i.e. those obtained after the second CsCl-gradient-centrifugation (CsCl II), were essentially free of all the enzyme activity associated with the crude soluble mucus. Although it was impossible to establish their identity, membranous structures and fuzzy electron-opaque particulate structures were noticeable on electron-microscopic examination of the negatively stained preparations at magnifications ranging from 20000 to 140000 (results not shown). On the other hand, neither was any marker-enzyme activity or electron-opaque material detectable in the highly purified mucin preparations. On average, the final yield of the highly purified mucin was 0.5% of the total starting material.

If mucosal scrapings were gently homogenized in an iso-osmolar buffer, centrifuged at 105000g for 60 min to remove microsomal fractions and other membranous organelles, and the procedure outlined in Scheme 1 was followed to purify the high- M_r mucus glycoprotein, its final yield was decreased substantially to less than 0.10% of the starting crude soluble mucus. However, its carbohydrate/protein ratio and the M_r were similar to those of the highly purified mucin described in the present investigation.

Table 1. Total protein and nucleic acid content of mucus preparations at various stages of purification
Results from one representative experiment are shown. For experimental details see Scheme 1 and the text.

Preparation	Total protein (mg)	Total DNA (mg)	RNA (mg)
Crude soluble mucus	500	43.5	11.40
Protamine sulphate treatment	451*	7.2	2.25
Deoxyribonuclease and ribonuclease treatment	443*	Not detectable	0.37
Partially purified mucin CsCl I	30.4	Not detectable	0.12
Partially purified mucin CsCl II	10.8	Not detectable	0.06
Highly purified mucin	1	Not detectable	0.0035

* Not corrected for contribution caused by protamine sulphate or added nucleases.

Fig. 1. Profile representing *CsCl*-density-gradient centrifugation of sheep small-intestinal crude soluble mucus. For details see the Experimental section and Scheme 1. ■, Total protein; △, total carbohydrate; ●, carbohydrate/protein ratio.

Fig. 2. Profile representing *CsCl*-density-gradient centrifugation of the pooled and concentrated higher-density fractions (5–7) obtained by the *CsCl*-density-gradient centrifugation of the crude soluble mucus as in Fig. 1. For details see the Experimental section and Scheme 1. ■, Total protein; △, total carbohydrate; ●, carbohydrate/protein ratio.

Immunological analysis

Antibodies against the highly purified mucin were produced in guinea pigs because they were found to be better responders than rabbits and mice. On immunoelectrophoresis, the highly purified

Fig. 3. H.p.l.c. of (a) partially purified mucin (b) and the high- M_r mucus glycoprotein obtained by h.p.l.c. of the leading peak isolated by h.p.l.c. of the partially purified mucin (see Scheme 1)

The h.p.l.c. was carried out in an LKB Blue column equilibrated with 0.2M-ammonium acetate, pH 6.0, and the V_0 was determined with giant-keyhole-limpet haemocyanin. Abbreviations: D.S., detector sensitivity; V_0 , void volume.

mucin yielded a single precipitin line, which migrated anodally (results not shown). However, no precipitin line was visible when the antigen used was crude soluble mucus. This could be due either to the presence of mucin in concentrations lower than that required for visible precipitation or to masking of the antigenic sites by the non-covalently bound proteins, subsequently removed during the process of purification. On Ouchterlony gel-diffusion analysis, only a single precipitin line was observed as well. Both the partially purified and the highly purified mucins were free of immunoglobulins, as judged by their lack of reactivity with anti-(normal sheep serum) and anti-(sheep IgG1), anti-(sheep IgG2), anti-(sheep IgM) and anti-(sheep IgA).

Immunofluorescence and tissue distribution of mucin

Indirect immunofluorescence analysis was carried out to determine the cellular and regional distribution of highly purified mucin in the gastrointestinal tract. Clusters of goblet cells, which were most numerous in the abomasum, caecum and colon, fluoresced as strongly as the mucus blanket. The fluorescence patterns observed for abomasum and caecum are shown (Figs. 4a and 4b); similar patterns were observed in other regions. Parallel staining of replicate tissue sections by the periodate/Schiff method revealed that the areas that showed immunofluorescence also stained strongly positive with the periodate/Schiff reagent (Figs. 4c and 4d).

Ultracentrifugal analysis

The sedimentation-velocity analysis of the highly purified mucin revealed only a single boundary on ultracentrifugation in either 0.32 ST (40S) or 5M-guanidinium chloride, pH 8.0 (20S) (Fig. 5).

Table 2. Distribution of some marker enzymes in various fractions during the purification of sheep small-intestinal goblet-cell mucin

The results are expressed as means $\pm$ S.D. for duplicate determinations on two different preparations obtained during purification of the high- M_r sheep mucin (see Scheme 1).

Organelle	Marker enzyme	Enzyme specific activity (μ mol/h per mg of protein)				Highly purified mucin
		Crude soluble mucus	Partially purified mucin		CsCl-gradient top	
			CsCl I	CsCl II		
Cytoplasm	Lactate dehydrogenase	4.00 $\pm$ 0.27	0.86 $\pm$ 0.04	0.02 $\pm$ 0.00	3.96 $\pm$ 0.25	0
Plasma membrane	Alkaline phosphatase	2.97 $\pm$ 0.02	0.60 $\pm$ 0.07	0.03 $\pm$ 0.01	2.74 $\pm$ 0.01	0
Mitochondria	Glutamate dehydrogenase	0.076 $\pm$ 0.002	0.183 $\pm$ 0.02	0.06 $\pm$ 0.02	0.052 $\pm$ 0.002	0
Endoplasmic reticulum	Glucose-6-phosphatase	0.006 $\pm$ 0.002	0	0	0.007 $\pm$ 0.002	0
Lysosomes	β -N-Acetylglucosaminidase	1677 $\pm$ 22	61.42 $\pm$ 0.39	0.04 $\pm$ 0.01	1185 $\pm$ 9	0
	Total protein (mg)	540	30.50	12.10	440*	1.0

* Not corrected for contribution caused by protamine sulphate or added nucleases.

Fig. 4. Indirect immunofluorescence assay for representative tissue sections from abomasum (a) and caecum (b) (magnification $160\times$)

The antisera used for development were guinea-pig anti-(highly purified mucin) antiserum and goat anti-(guinea-pig IgG)-fluorescein isothiocyanate conjugate. Also shown are representative periodate/Schiff-stained sections of abomasum (c) and caecum (d) (magnification $160\times$).

Sedimentation-equilibrium analysis of the highly purified mucin was carried out at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml in 0.32 ST and in 0.32 ST in $^2\text{H}_2\text{O}$ at 0.5 mg/ml. The partial specific volume ($\bar{v}$) calculated at the last system was $0.686\text{cm}^3/\text{g}$ (Fig. 6) and was assumed to be the same for all concentrations of the highly purified mucin. That reported previously for the sheep submaxillary mucin was 0.663 (Hill *et al.*, 1977). The

weight-average M_r of the highly purified mucin was calculated to be $5(\pm 0.1) \times 10^6$ and was found to be concentration-independent (results not shown). This is in contrast with the concentration-dependence reported for the sheep submaxillary mucin (Gottschalk & McKenzie, 1961; Hill *et al.*, 1977) and bovine and pig mucins (Payza *et al.*, 1970). The M_r of the highly purified sheep intestinal mucin was greater than that reported for

Fig. 5. Schlieren pattern of the highly purified mucin in 5M guanidinium chloride, pH 8.0

The photograph was taken 44 min after reaching a speed of 42040 rev./min.

Fig. 6. Sedimentation-equilibrium data for highly purified sheep high- M_r mucus glycoprotein in buffer (0.32 ST) (■) and in buffer in $^2\text{H}_2\text{O}$ (●)

The lines were fitted by the least-squares method.

the sheep submaxillary mucin (Gottschalk *et al.*, 1972; Hill *et al.*, 1977), pig intestinal mucus glycoprotein (Mantle & Allen, 1981), rat intestinal mucin (Forstner *et al.*, 1973b) and human intestinal mucin (Jabbal *et al.*, 1976; Forstner *et al.*, 1979). However, it was smaller than that reported for the pig colonic mucin (Marshall & Allen, 1978). The difference observed between the M_r values of pig intestinal and colonic mucus glycoproteins (1.7×10^6 and 15×10^6 respectively) suggested that the two types of macromolecules were different in their quaternary structures. Although we do not have comparable data because we did not purify mucin from regions other than the sheep small intestine, indirect immunofluorescence indicated that the mucins from abomasum, small intestine, caecum and colon were at least antigenically similar. Similar results were reported for the human intestinal goblet-cell mucin, whereby mucins from the rectum, colon and small intestine were found to have identical affinity for rabbit antibody, although that from the stomach reacted less strongly (Qureshi *et al.*, 1979).

SDS/polyacrylamide-gel electrophoresis

The purpose of performing SDS/polyacryl-

amide-gel electrophoresis in 3.3% gels in this investigation was not to demonstrate homogeneity of the highly purified mucin preparations but to demonstrate freedom from lower- M_r contamination. In our hands, the SDS/polyacrylamide-gel electrophoresis methods reported to be suitable for resolution of high- M_r macromolecules unfortunately either were not completely reproducible (Perret *et al.*, 1983) or were found to be inapplicable (Peacock & Dingman, 1968).

When the crude soluble mucus was subjected to SDS/polyacrylamide-gel electrophoresis and stained with Coomassie Blue or Kenacid R, at least six bands, with M_r values <56000 (one band), 62000, 105000, 136000, 280000 and >280000 (one band) respectively, were detectable (results not shown). Staining of a replicate gel with periodate/Schiff reagent also revealed a minimum of six bands, but with M_r values <56000 (one band), 280000 (one band) and >280000 (four bands) respectively (results not shown). When the partially purified mucin was subjected to SDS/polyacrylamide-gel electrophoresis and the gel stained with Kenacid R, no bands were visible. However, on staining with periodate/Schiff reagent four bands with M_r values greater than 280000 were observed (results not shown). On SDS/polyacrylamide-gel electrophoresis of highly purified mucin, only a single band near the top of the gel was seen after staining by the periodate/Schiff method or the silver-staining procedure, but it could not be stained with either Coomassie Blue or Kenacid R. Thus it is clear that the highly purified mucin is a carbohydrate-rich glycoprotein, as are other mucus glycoproteins investigated, and is free of the contaminating lower- M_r components that were present in the partially purified mucin preparations. However, in spite of the observed apparent monodispersity of the highly purified mucin on sedimentation-velocity and sedimentation-equilibrium analysis, homogeneity of this preparation cannot be claimed to have been rigorously demonstrated.

Chemical analysis

The results of chemical analyses are summarized in Tables 3 and 4. That the CsCl-density-gradient centrifugation of the crude soluble mucus resulted in mucin enrichment in the higher-density fractions was clear from the observed increase in the average carbohydrate/protein ratios (Table 3). The highly purified mucin was found to consist of approx. 66% carbohydrate and 34% of protein (w/w). Although the peaks II–IV (Fig. 3a) emerging from the h.p.l.c. of the partially purified mucin (CsCl II) were neither further purified nor identified, the carbohydrate/protein ratio of the combined peaks was 4:7 (w/w).

Table 3. Carbohydrate and protein contents of various preparations during purification of the high-*M_r* small-intestinal mucin

Results shown represent biochemical determinations of various mucin preparations in four separate experiments and are presented as means $\pm$ S.D.

Preparation	Content (%)		
	Carbohydrate	Protein	Yield (%)*
I Crude soluble mucus	2.3 $\pm$ 1.5	95 $\pm$ 6	100
II Glycoprotein fraction CsCl I	21 $\pm$ 3	79 $\pm$ 5	7 $\pm$ 1
III Glycoprotein fraction CsCl II†	50 $\pm$ 2	50 $\pm$ 2	4.4 $\pm$ 0.3
IV Highly purified mucin	65.8 $\pm$ 0.4	34.2 $\pm$ 0.5	0.5 $\pm$ 0.1

* Expressed as percentage of total dry weight.
† Also referred to as partially purified mucin.

Table 4. Chemical composition of the highly purified small-intestinal mucus glycoprotein

All analytical determinations were made in quadruplicate on four separate preparations of the highly purified mucin, and are presented as means $\pm$ S.D.

Composition (% by wt.)	
Total carbohydrate	66
Total protein	34
Neutral hexoses	30.5 $\pm$ 0.29
Sialic acid	1.4 $\pm$ 0.10
Fucose	0.5 $\pm$ 0.08
Glucosamine	11.5 $\pm$ 1.08
Galactosamine	16.1 $\pm$ 0.56
Sulphate	7.1 $\pm$ 0.60

Neutral sugars accounted for 30.5% and glucosamine and galactosamines for 13% and 18% respectively of the total weight. Whereas fucose constituted 0.54% of the highly purified mucin, sialic acid was 1.4%. In contrast, the sialic acid content of the purified sheep submaxillary mucin was reported to be 34.3% (Hill *et al.*, 1977). The very small amount of sialic acid detected in our highly purified small-intestinal mucin cannot be attributed to its removal by neuraminidase, because all the buffers or salts used in this investigation at the prescribed H⁺ concentrations had previously been found to be completely inhibitory to neuraminidase activity (T. K. S. Mukkur, unpublished work). Sulphate content was estimated to be 7.1% (w/w), thus suggesting that the high-*M_r* small-intestinal mucus glycoprotein was a sulphated glycoprotein. Previously, a sialoprotein subunit with *M_r* approx. 100000 isolated from sheep colonic mucin (prepared by a method not involving CsCl-density-gradient centrifugation and h.p.l.c.) was reported to be sulphated (Kent & Marsden, 1963).

Amino acid composition

The highly purified mucus glycoprotein was hydrolysed in 4*M*-methanesulphonic acid contain-

Table 5. Amino acid composition of the small-intestinal mucus glycoprotein

Amino acid composition was determined as described in the Experimental section and is expressed as the means $\pm$ S.D. for duplicate determinations on two different preparations of the highly purified mucin.

Amino acid	Content (nmol/mg of protein)
Lysine	117 $\pm$ 6
Histidine	Trace
Ammonia	4020 $\pm$ 152
Arginine	102 $\pm$ 3
Aspartic acid	365 $\pm$ 10
Threonine	1440 $\pm$ 12
Serine	415 $\pm$ 5
Glutamic acid	414 $\pm$ 10
Proline	535 $\pm$ 11
Glycine	314 $\pm$ 7
Alanine	271 $\pm$ 7
Half-cystine	548 $\pm$ 9
Valine	1425 $\pm$ 17
Methionine	Trace
Isoleucine	95 $\pm$ 3
Leucine	202 $\pm$ 7
Tyrosine	85 $\pm$ 8
Phenylalanine	95 $\pm$ 5

ing 0.2% tryptamine (Simpson *et al.*, 1976), and the amino acid composition was determined in a Beckman amino acid analyser (Table 5). Valine and threonine were the predominant amino acids in the highly purified small-intestinal mucin. The next most abundant amino acids were half-cystine and proline, followed by glutamic acid and serine. This was substantially different from the amino acid composition of sheep submaxillary mucin (Hill *et al.*, 1977), which has been reported to be devoid of cysteine, methionine, tyrosine and tryptophan, and of which about 75% of the composition can be accounted for by threonine, serine, proline, glycine and alanine. It is strongly suggestive, then, that mucins from different physiological systems of sheep may be at least

structurally and possibly functionally different from each other, as has been suggested for the human mucus glycoproteins (Qureshi *et al.*, 1979).

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