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# The disulphide-bond content and rheological properties of intestinal mucins from normal subjects and patients with cystic fibrosis

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The disulphide/thiol (S–S/SH) content and rheological properties of highly purified small-intestinal mucins from normal (N) subjects and patients with cystic fibrosis (CF) were investigated. (1) An assay was developed to measure free SH groups (before reduction) and total SH content (after reduction) using 4,4′-dipyridyl disulphide. S–S bonds were calculated by difference. Experimental values for the S–S and SH contents of well-characterized proteins obtained with the assay showed good agreement with expected values. (2) The S–S and free SH contents of nine N and six CF mucins were variable: 44.4 ± 5.4 nmol of S–S and 4.3 ± 1.1 nmol of free SH per mg of N mucin and 31.7 ± 7.6 nmol of S–S and 7.5 ± 3.7 nmol of SH per mg of CF mucin. N and CF mucins were not statistically different. In most mucins, ~ 90 % of the SH groups were involved in S–S bonds. (3) Gels were reconstituted from the same N and CF mucins at concentrations between 8 and 25 mg/ml and their rheological properties were assessed by using a magnetic microrheometer. (4) Once formed, mucin gels were stable and maintained the same mechanical properties over a long period of time (3–14 days). (5) The rheological profiles of both N and CF samples did not vary with the concentration of mucin present and were characteristic of weak, visco-elastic gels. (6) Although variations were seen in the visco-elastic properties of individual mucins, no systematic differences were detected between N and CF preparations. (7) There was no apparent correlation between the rheological properties of a mucin and its S–S/SH content.

## INTRODUCTION

Mucus secretions in CF are frequently described as 'thick' and 'tenacious', and many of the clinical problems associated with the disease are directly or indirectly caused by the accumulation of large amounts of mucus in the respiratory, gastrointestinal and cervical tracts. Although mucus secretions throughout the body appear abnormal in CF, it has proven difficult to find a consistent, specific defect in either the epithelial cells that produce mucus or the mucus glycoproteins (mucins) that constitute the major gel-forming component of the secretion. In the large intestine, one study reported that goblet cells were more 'prominent' in CF, containing copious amounts of strongly stained mucus (Johansen & Kay, 1969), whereas another study indicated that the number of goblet cells and their content of intracellular mucus were normal in CF (Neutra & Trier, 1978). No histochemical change was observed in rectal goblet-cell mucin in CF (Johansen & Kay, 1969) and no abnormality was detected in mucus glycoprotein synthesis, transport and secretion in the CF rectal mucosa (Neutra *et al.*, 1977). In contrast, small-intestinal goblet cells are apparently unchanged in size and number in CF, but mucins are altered histochemically (showing an increase in sulphation; Jeffrey *et al.*, 1983; Morrissey & Tymvios, 1978; Park *et al.*, 1987), mucin synthesis and secretion are elevated (Forstner *et al.*, 1981) and the mucus layer appears thicker (Freye *et al.*, 1964; Jeffrey *et al.*, 1983). Since there is no consistent change in goblet cells, mucin histochemistry or mucin synthesis and secretion, it seems possible that the factors contributing to mucus accumulation in CF may vary among different tissues and organs.

Biochemical studies have attempted to find changes at the molecular level in isolated CF mucin. Small-intestinal mucin purified from CF patients has a higher sulphate content than normal mucin and is more heavily glycosylated, apparently due to extension of the oligosaccharide side chains by additional galactose, *N*-acetylglucosamine and fucose residues (Wesley *et al.*, 1983*a*,*b*). Recently, we noted that the immunoreactivity of CF mucin with an antibody directed against the 118 kDa 'link' glycoprotein was generally greater compared with that of normal mucin (Mantle & Stewart, 1989). Enhanced immunoreactivity was not due to an increase in the 118 kDa glycoprotein content of CF mucin or to an increase in the number of antigenic determinants within the CF 118 kDa glycoprotein itself. It was therefore suggested that the accessibility of the 118 kDa glycoprotein for antibody binding may be greater in CF mucin than in normal mucin, possibly as a result of a difference in their three-dimensional conformations (Mantle & Stewart, 1989). Whether additional carbohydrate and sulphate residues are responsible for an altered conformation in CF mucin has yet to be determined.

Many gastrointestinal mucins have similar polymeric

Abbreviations used: N, normal; CF, cystic fibrosis; $Nbs_2$, 5,5′-dithiobis-(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; 4,4′-PDS, 4,4′-dipyridyl disulphide.

structures, composed of large, heterogeneous glycoprotein monomers and a smaller discrete 'link' (glyco)protein ($M_r$ 70000–120000) held together by disulphide bonds (Mantle *et al.*, 1981, 1984*a*,*b*; Pearson *et al.*, 1981; Fahim *et al.*, 1983; Mantle & Thakore, 1988). Proteolysis or thiol reduction depolymerizes these mucins, resulting in a loss of their visco-elastic properties and solubilization of the mucus gel (Bell *et al.*, 1985; Sellers *et al.*, 1988). Since disulphide bonds are important for maintaining the macromolecular structure of mucins, we wondered whether an alteration in the disulphide-bond content of CF mucins caused the conformational change noted previously (Mantle & Stewart, 1989) and subsequently affected the gel-forming properties of mucin in CF. The present study was therefore designed to compare the disulphide-bond content and rheological properties of normal and CF mucins.

## METHODS

### Isolation and purification of mucin

Human small intestine was obtained within 12 h *post mortem* from normal (N) subjects with no history of gastrointestinal disease and from patients with cystic fibrosis (CF). Mucin was purified from mucosal homogenates in the presence of proteolytic inhibitors [5 mM-$Na_2EDTA$, 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 10 mM-*N*-ethylmaleimide (NEM)] by equilibrium-density-gradient centrifugation in CsCl (twice), followed by gel filtration on Sepharose-CL 2B (Pharmacia Fine Chemicals, Uppsala, Sweden). High-molecular-mass polymeric mucin was harvested from the void-volume fractions of the column (Mantle *et al.*, 1984*a*,*b*). The absence of non-mucin protein bands on SDS/PAGE and the failure to detect DNA (Hinegardner, 1971), mannose, glucose and uronic acid (by g.l.c.) established the purity of all mucin preparations. Previous studies have shown that mucin purified as described contains less than 5% (w/w) of lipid (Mantle & Forstner, 1986). Three mucin preparations (GM, JK and JR, kindly supplied by Dr. J. F. Forstner and Dr. G. G. Forstner, Hospital for Sick Children, Toronto, Canada) were purified by gel filtration of mucosal homogenates on Sepharose-CL 4B, followed by chromatography of the void-volume fractions on Sepharose-CL 2B and recovery of the excluded and partially included mucin peaks (Wesley *et al.*, 1983*b*). Many of the mucins analysed in the present studies were used previously for immunological and biochemical investigations on their 118 kDa glycoprotein (Mantle & Stewart, 1989).

### Determination of free SH groups and S–S bonds

The conditions for the assay of S–S and SH groups were determined from the earlier work of Grassetti & Murray (1967), Habeeb (1972) and Riddles *et al.* (1983). To measure total SH content, purified mucin (0.5 mg, dried to constant weight over $P_2O_5$ and KOH *in vacuo*) was reduced with 0.33 M-$NaBH_4$ in 8 M-urea/20 mM-$Na_2EDTA$/0.1 M-$NaH_2PO_4$/$Na_2HPO_4$, pH 9 (final volume of 2 ml) at 37 °C for 90 min. One drop of octan-1-ol was added to the reducing solution to prevent foaming. After adding 300 $\mu$l of 20% (w/v) SDS, excess $NaBH_4$ was destroyed by titrating the solution to pH 5.4 with acetic acid (addition of 250 $\mu$l of a 5 M solution) and incubating the mixture at 37 °C for 15 min. After addition of 4,4′-dipyridyl disulphide (4,4′-PDS; Sigma Chemical Co., St. Louis, MO, U.S.A.; 200 $\mu$l of a 2 mM solution in 0.2 M-sodium acetate, pH 5) and incubation for 30 min at room temperature (21–23 °C), the absorbance of the solution was measured at 324 nm against a blank containing all reagents except mucin. The total SH content of the mucin was calculated by using a molar absorption coefficient of 19800 $M^{-1} \cdot cm^{-1}$. Experimental values are expressed per mg of mucin, per mg of mucin protein or per nmol of serine plus threonine in the mucin preparation.

Free SH groups (i.e. those not associated in S–S bonds) were determined by using 1 mg (dry weight) of mucin. Samples were dissolved in 1.6 ml of 20 mM-$Na_2EDTA$/2% (w/v) SDS/0.2 M-sodium acetate, pH 5.4, and incubated at 37 °C for 15 min. After addition of 200 $\mu$l of 2 mM-4,4′-PDS (as above) and incubation at room temperature for 30 min, the absorbance of the solution was measured at 324 nm against a reagent blank. The free SH content of the mucin was determined by using the same molar absorption coefficient as above. Experimental values are again expressed per mg of mucin, per mg of mucin protein or per nmol of serine plus threonine in the mucin preparation. The S–S bond content of a mucin (in nmol) was calculated as the total amount (nmol) of SH minus the amount (nmol) of free SH groups.

All assays were carried out in duplicate. For each step of the procedure, optimal conditions of time, temperature, pH and concentration of reagents were established. Fresh $NaBH_4$ and 4,4′-PDS solutions were used for each assay. The reliability of the assay was assessed by analysis of standard proteins with known S–S and SH contents; these proteins were bovine pancreatic RNAase, $\alpha$-chymotrypsinogen A, lysosyme, human serum albumin and trypsinogen (Sigma Chemical Co.).

### Chemical analyses

Amino acids were quantified on a Beckman 6300 amino acid analyser (Beckman Instruments, Palo Alto, CA, U.S.A.) after hydrolysis for 24 h at 110 °C in 6 M-HCl *in vacuo*. Carbohydrates were quantified by g.l.c. (Chaplin, 1982).

### Rheological analyses

Gels were reconstituted in phosphate-buffered Ringer solution, pH 7.0, from freeze-dried mucin at various concentrations between 8 and 25 mg/ml. A small quantity (1–2 mg) of mucin was carefully weighed in a sealable 1.5 ml polypropylene tube and covered with an appropriate volume of solvent (50–150 $\mu$l). To ensure complete solubilization of the mucin, gels were allowed to form at 4 °C for at least 3 days before rheological assessment. Homogenization and shearing of the mucin gels was strictly avoided. In some cases, gels were assessed for their rheological properties at 3, 6, 10 and 13 days after reconstitution; the gels were stored at 4 °C until analysed, and separate aliquots were used on each occasion.

The visco-elastic properties of mucin gels were measured in a magnetic microrheometer (King, 1988). A small steel sphere ($\sim$ 100 $\mu$m in diameter) was inserted in a 5–10 $\mu$l aliquot of gel and vibrated by means of an electromagnet over a frequency range of 1–100 rad/s. The amplitude of the vibration and the phase lag with respect to the driving force were used to calculate the elasticity and viscosity at each driving frequency (King, 1988). All

experiments on the microrheometer were carried out at room temperature (21–23 °C) and under conditions of low strain (0–10%). The rheological parameters determined by the magnetic microrheometer are: $G'$, the storage modulus (characteristic of elastic or solid properties) expressed in Pa; $G''$, the loss modulus (characteristic of viscous or liquid properties) expressed in Pa; and $\tan\delta$, the loss tangent, the ratio of viscous/elastic ($G''/G'$) response at any given frequency. The inter-aliquot variability in $G'$ and $G''$ was ~40% or 0.15 log units, whereas the variability in $\tan\delta$ was ~15%.

## RESULTS

### Determination of S–S and SH groups

In the past a number of assays for free SH groups in proteins have been developed on the basis of their reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) ($Nbs_2$; Ellman's reagent; Habeeb, 1972; Riddles *et al.*, 1983). There are, however, a variety of potential problems that may arise when using $Nbs_2$, since: (1) $Nbs_2$ reacts with SH groups at different rates in different proteins and, in some cases, especially with 'buried' SH groups, reaction can be slow; (2) the absorption coefficient of $Nbs_2$ can vary at different temperatures and pH values and in the presence of particular salts and detergents; (3) the absorption maximum of the $Nbs_2$–protein complex occurs at pH 8, but free $Nbs_2$ is degraded at this pH into absorbing compounds that can then interfere with SH determinations; and (4) the $Nbs_2$–protein complex is oxidized by molecular $O_2$ and is therefore unstable over long time periods unless it is maintained in an $O_2$-free environment (Grassetti & Murray, 1967; Riddles *et al.*, 1983). To overcome these problems, highly purified $Nbs_2$ is required and assays must be conducted at non-optimal pH under positive $N_2$ pressure. Furthermore, care is needed to ensure that reaction of $Nbs_2$ and free SH groups in the protein under investigation is complete before any side reactions producing interfering substances can occur. In contrast, Grassetti & Murray (1967) found

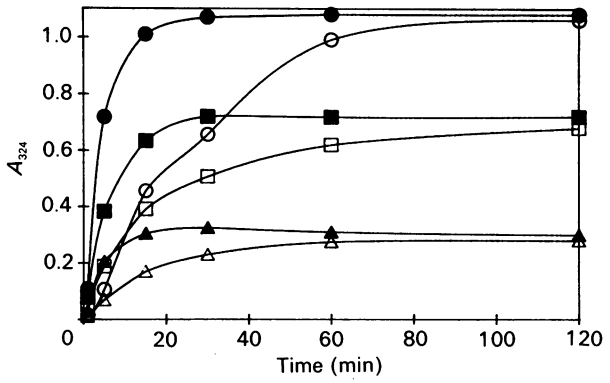


**Fig. 1. Time- and temperature-dependence of reduction in the S–S/SH assay**

Human small-intestinal mucin (H35; 0.4 mg dry wt.), human serum albumin (0.2 mg) and ribonuclease (0.2 mg) were analysed for total SH content by the standard assay procedure, but the reduction step was carried out for various lengths of time at either 37 °C (closed symbols) or room temperature (21–23 °C; open symbols). △, ▲, Mucin; □, ■, serum albumin; ○, ●, ribonuclease.

**Table 1. S–S and SH content of standard proteins**

Each protein (0.2 mg) was analysed at least six times in the S–S/SH assay. Free SH groups (not disulphide-bound) were measured before reduction and total SH content was determined after reduction. The amount (nmol) of SH groups involved in S–S bonds was calculated by difference. The number of free SH groups and S–S bonds per molecule was then calculated from the known molecular mass of the protein. Each experimental value represents the mean ± S.E.M..

| | Experimental | | Expected | |
|---|---|---|---|---|
| | No. of S–S bonds per molecule | No. of free SH groups per molecule | No. of S–S bonds per molecule | No. of free SH groups per molecule |
| Ribonuclease | 7.4±0.13 | 0 | 8.0 | 0 |
| α-Chymotrypsinogen | 7.5±0.05 | 0 | 10.0 | 0 |
| Trypsinogen | 12.7±0.2 | 0 | 12.0 | 0 |
| Lysozyme | 7.8±0.1 | 0 | 8.0 | 0 |
| Human serum albumin | 33.0±0.45 | 0.43±0.03 | 34.0 | 0.5 |

**Table 2. S–S and SH content of N and CF intestinal mucins**

Small intestine was obtained from N subjects and patients with CF. 'Time (h)' refers to the time *post mortem* that the intestinal tissue was acquired. Mucins were purified from mucosal homogenates by equilibrium-density-gradient centrifugation in CsCl and gel filtration on Sepharose-Cl 2B. 'Protein (%)' refers to the mucins' protein contents determined from amino acid analyses. Free SH content was measured before reduction, whereas total SH content was determined after reduction. The amount of S–S (in nmol) was calculated by difference. Each mucin was analysed at least twice in duplicate.

| Mucin | | Time (h) | Protein (%) | Content (nmol/mg of mucin) Total SH | Free SH | S–S |
|---|---|---|---|---|---|---|
| N | H13 | 4 | 7.0 | 22 | 3 | 19 |
| | H14 | 4 | 12.4 | 34 | 3 | 31 |
| | H11 | 12 | 15.6 | 39 | 4 | 35 |
| | H21 | 5.5 | 12.0 | 45 | 3 | 42 |
| | H24 | 5 | 14.6 | 45 | 2 | 43 |
| | H35 | 4.5 | 10.0 | 45 | 2 | 43 |
| | H25 | 7 | 13.0 | 54 | 3 | 51 |
| | H20 | 7 | 19.5 | 70 | 7 | 63 |
| | GM | 3 | 18.0 | 85 | 12 | 73 |
| | Average... | 5.8 | 13.6 | 48.8 | 4.3 | 44.4 |
| | ±S.E.M.... | 0.9 | 1.3 | 6.3 | 1.1 | 5.4 |
| CF | CF2 | 7 | 20.0 | 21 | 2 | 19 |
| | CF4 | 7 | 7.0 | 21 | 1 | 20 |
| | CF1 | 11 | 18.4 | 36 | 3 | 33 |
| | JK | 2 | 15.0 | 39 | 23 | 16 |
| | JR | 12 | 18.0 | 50 | 14 | 36 |
| | CF3 | 5.5 | 13.5 | 68 | 2 | 66 |
| | Average... | 7.4 | 15.3 | 39.2 | 7.5 | 31.7 |
| | ±S.E.M.... | 1.5 | 1.9 | 7.3 | 3.7 | 7.6 |

**Table 3. S–S and SH content of N and CF intestinal mucins, expressed relative to their protein and serine plus threonine content**

Purified mucins from the small intestine of N subjects and patients with CF were analysed for their free SH content before reduction and their total SH content after reduction. The amount (nmol) of S–S was calculated by difference. The protein composition and content of mucin preparations was determined from amino acid analyses. Total SH and S–S data are expressed per mg of mucin protein and per nmol of serine plus threonine (Ser+Thr) in the mucin preparation. Each mucin was analysed at least twice in duplicate.

| | Mucin | Content (nmol/mg) | | Content (nmol/nmol of Ser+Thr) | |
|---|---|---|---|---|---|
| | | Total SH | S–S | Total SH | S–S |
| N | H13 | 314 | 271 | 0.076 | 0.066 |
| | H14 | 274 | 250 | 0.069 | 0.063 |
| | H11 | 250 | 224 | 0.060 | 0.054 |
| | H21 | 375 | 350 | 0.090 | 0.084 |
| | H24 | 308 | 294 | 0.078 | 0.074 |
| | H35 | 450 | 430 | 0.107 | 0.102 |
| | H25 | 415 | 392 | 0.099 | 0.094 |
| | H20 | 359 | 323 | 0.090 | 0.081 |
| | GM | 472 | 406 | 0.143 | 0.123 |
| | Average... | 357.4 | 326.7 | 0.090 | 0.082 |
| | ±S.E.M.... | 25.9 | 24.3 | 0.008 | 0.007 |
| CF | CF2 | 105 | 95 | 0.022 | 0.020 |
| | CF4 | 300 | 285 | 0.077 | 0.073 |
| | CF1 | 196 | 179 | 0.065 | 0.060 |
| | JK | 260 | 107 | 0.080 | 0.033 |
| | JR | 278 | 200 | 0.086 | 0.062 |
| | CF3 | 503 | 489 | 0.127 | 0.123 |
| | Average... | 273.7 | 225.8 | 0.0076 | 0.062 |
| | ±S.E.M.... | 54.2 | 59.7 | 0.014 | 0.015 |

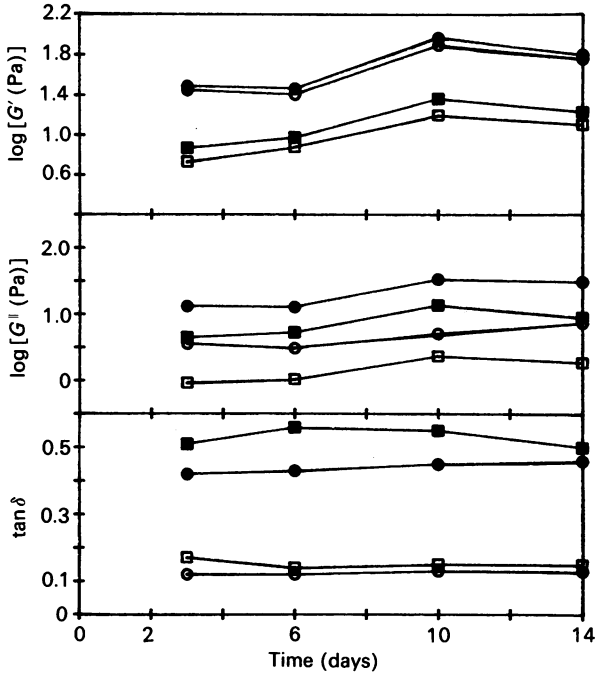


**Fig. 2. Rheological properties of intestinal mucin gels at various times after reconstitution**

Gels were reconstituted from freeze-dried mucins in phosphate-buffered Ringer solution pH 7.0, at a concentration of 20 mg/ml. After incubation at 4 °C for the time periods shown, the rheological properties of the gels were determined at room temperature on a magnetic microrheometer over a frequency range of 1–100 rad/s. Separate gels were analysed at each time point. $G'$ is the storage modulus, $G''$ is the loss modulus and $\tan\delta$ is the ratio of $G''/G'$. Open symbols represent values at 1 rad/s; closed symbols represent values at 100 rad/s. □, ■, H35; ○, ●, CF3.

that 4,4′-PDS reacted rapidly with all free SH groups in proteins to produce a stable complex with an absorption coefficient that did not vary over a wide pH range and was not susceptible to changes in temperature, salt and detergent concentration. Therefore, in developing our assay, we incorporated some of the features of older $Nbs_2$-based procedures and utilized 4,4′-PDS to detect SH groups.

Initially, the time- and temperature-dependence of the reduction step of the assay was investigated by incubating mucin samples for various times (up to 150 min) in the $NaBH_4$ reducing solution at room temperature or 37 °C before reaction with 4,4′-PDS. Reduction was essentially complete at both temperatures by 60 min (Fig. 1). For convenience and always to ensure complete reduction, subsequent assays were carried out by using 90 min reductions at 37 °C.

The optimal concentration of 4,4′-PDS required for the assay was also determined. Although the amount of 4,4′-PDS added had little effect on net absorption (i.e. sample minus reagent blank), concentrations of 4,4′-PDS between 4 and 10 mM produced high values for the reagent blanks, between 0.2 and 0.4 when read against water, compared with < 0.08 for reagent blanks to which 2 mM-4,4′-PDS had been added. Since a high absorption in the reagent blank may influence the accuracy of the assay, a final concentration of 2 mM-4,4′-PDS was selected for the standard procedure. In investigating the time required for the formation of the PDS–mucin complex and its stability, we established that reaction was complete in less than 10 min at room temperature and that the absorption of the complex was unchanged up to 70 min. Finally, to improve S–S cleavage, prevent refolding of protein and ensure complete binding of 4,4′-PDS to SH groups, denaturants (urea and/or SDS) were present throughout the assay procedure. To assess the reliability of the assay, various standard proteins of known S–S and SH content were analysed (Table 1). The experimental data obtained showed good correlation with expected values (< 8% difference, with the exception of α-chymotrypsinogen A, which consistently produced a 25% difference between experimental and expected values).

### S–S/SH content of N and CF mucins

The S–S and SH contents of 15 purified human intestinal mucins (nine N and six CF samples) are shown in Table 2. No differences were observed between N and CF mucins with respect to either the time *post mortem* that the intestinal tissue was obtained (3–12 h for N

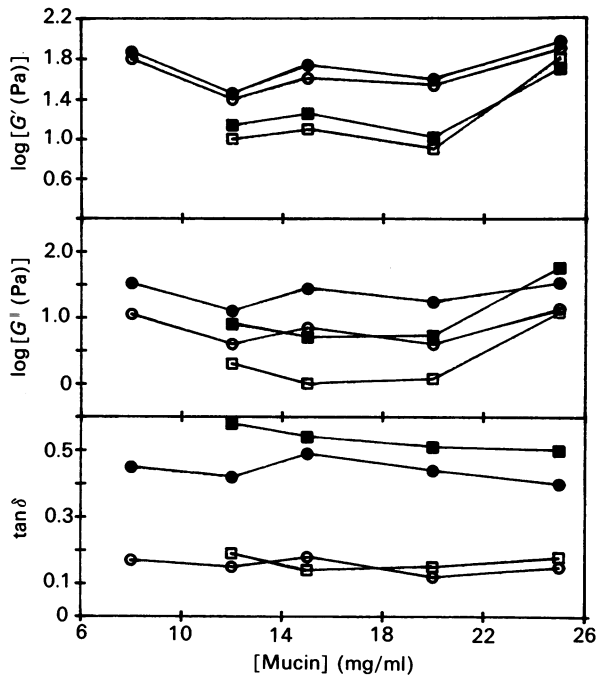


**Fig. 3. Concentration-dependence of the rheological properties of intestinal mucin gels**

Mucin gels were reconstituted in phosphate-buffered Ringer solution, pH 7.0, at concentrations between 8 and 25 mg/ml. After incubation at 4 °C for 6–10 days, the rheological properties of the gels were determined at room temperature by using a magnetic microrheometer over a frequency range of 1–100 rad/s/. $G'$ is the storage modulus, $G''$ is the loss modulus and tan $\delta$ is the ratio $G''/G'$. Open symbols represent values at 1 rad/s; closed symbols represent values at 100 rad/s. ■, □, H35; ○, ●, CF3.

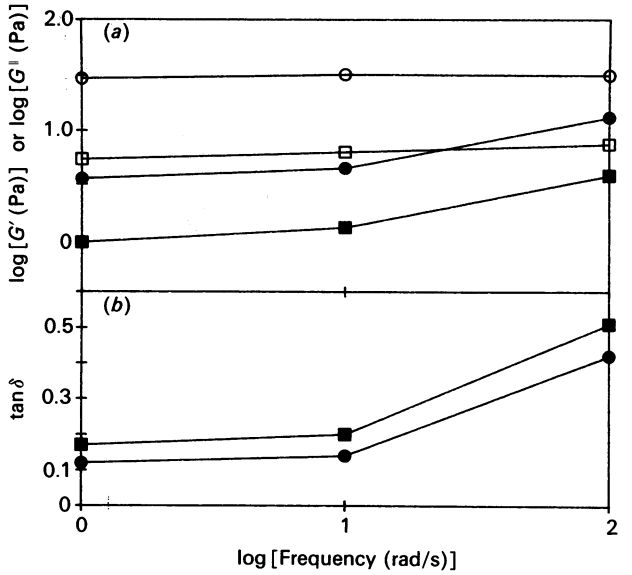


**Fig. 4. Frequency-dependence of the rheological properties of intestinal mucin gels**

Mucin gels were reconstituted at a concentration of 20 mg/ml in phosphate-buffered Ringer solution, pH 7.0, and their rheological properties were evaluated at room temperature by using a magnetic microrheometer over a frequency range of 1–100 rad/s. (*a*) $G'$ is the storage modulus (open symbols), $G''$ is the loss modulus (closed symbols); (*b*) tan $\delta$ is the ratio $G''/G'$. □, ■, H35; ○, ●, CF3.

tissue; 2–12 h for CF tissue) or the protein content of the purified mucins (7–20%, w/w, for both N and CF mucins). The protein content of individual N and CF mucin preparations was not apparently related to the time *post mortem* that the tissue was acquired. Although each mucin gave consistent values in the assay (< 15% inter-assay variation), considerable variability (3–4-fold) was observed among the individual preparations in their content of total SH, free SH and S–S groups when expressed relative to mucin dry weight (Table 2). Calculation of experimental data relative to the mucin protein content or per nmol of serine plus threonine in the mucin preparation did not eliminate the variability in the S–S/SH contents of the mucins (Table 3). Regardless of the manner in which S–S/SH content was expressed, no differences were detected between N and CF mucins ($P > 0.1$). The amounts (nmol) of total SH, free SH and S–S bonds (expressed per mg of mucin, per mg of mucin protein or per nmol of serine plus threonine) were not apparently related to the time *post mortem* that the small intestine was obtained, or to the mucin protein content ($P > 0.1$). Some samples acquired rapidly after death had lower S–S/SH contents than samples obtained at later times (e.g. H13 and H14 compared with H20 and H25). Similarly, some mucins with a relatively low protein content had more S–S/SH groups than mucins with a higher protein content (e.g. H24 and H35 compared with CF1 and CF2). From the total SH content of the mucins it appears that cysteine residues comprise between 1 (CF2) and 6% (GM and CF3) (w/w) of the mucin protein. In most cases, ~ 90% of the SH groups detected in the mucin were involved in S–S bonds. However, in two mucin preparations, namely JK and JR, known to contain Sepharose-CL 2B-partially-included (non-polymeric) material, a smaller proportion of the SH groups were S–S-bonded (41 and 72% respectively).

### Rheological properties of N and CF mucins

To determine the appropriate time for reconstitution of mucin gels, freeze-dried mucin samples were incubated in buffer at 4 °C and the rheological properties of the ensuing gels were measured after various periods of time (Fig. 2). Values obtained for $G'$, $G''$ and tan $\delta$ did not change appreciably between 3 and 14 days. For consistency in subsequent analyses, we allowed between 6 and 10 days for mucin gels to reconstitute before rheological assessment.

As illustrated in Fig. 3, $G'$, $G''$ and tan $\delta$ did not vary with the concentration of mucin present in the gel for either N or CF samples. However, some variability was observed in the concentration at which individual mucins formed a gel. For example, CF3 mucin gelled at 8 mg/ml, H35 did not gel until 12 mg/ml, and three mucins (H21, CF2 and JK) did not gel even at 25 mg/ml. At present, the reason for this variability in gelling concentration is not known.

The rheological properties of reconstituted mucin gels were determined over a frequency range of 1–100 rad/s (Fig. 4). For both N and CF mucin gels, $G'$ showed essentially no frequency dependence, although $G''$ and,

**Table 4. Rheological properties of N and CF intestinal mucins**

Purified freeze-dried mucins were suspended in phosphate-buffered Ringer solution, pH 7.0, at concentrations between 8 and 25 mg/ml. Gels were allowed to reconstitute for at least 6 days at 4 °C before analysis of their rheological properties at room temperature (21–23 °C) on a magnetic microrheometer. $G'$ (the storage modulus in Pa) and $G^{\parallel}$ (the loss modulus in Pa) were determined at frequencies between 1 and 100 rad/s. $\tan\delta$ is the ratio of $G^{\parallel}/G'$. Since the rheological properties of both N and CF intestinal mucin gels are independent of mucin concentration (see Fig. 3), all values of $G'$, $G^{\parallel}$ and $\tan\delta$ for a given mucin gel at various concentrations and at the two different frequencies were averaged. 'Fluid' means that the mucins formed viscous fluids but did not gel, even at the highest concentration (25 mg/ml) used in these studies.

| | Frequency (rad/s)... | 1 | | | 100 | | |
|---|---|---|---|---|---|---|---|
| | Mucin | log $G'$ | log $G^{\parallel}$ | tan $\delta$ | log $G'$ | log $G^{\parallel}$ | tan $\delta$ |
| N | H13 | 1.08 | 0.37 | 0.20 | 1.31 | 1.25 | 0.90 |
| | H11 | 2.06 | 1.27 | 0.16 | 2.25 | 2.11 | 0.73 |
| | H21 | | | Fluid | | | |
| | H24 | 1.96 | 1.13 | 0.15 | 2.03 | 1.83 | 0.64 |
| | H35 | 0.93 | 0.12 | 0.16 | 1.06 | 0.80 | 0.54 |
| | H25 | 1.91 | 0.96 | 0.11 | 1.91 | 1.60 | 0.49 |
| | H20 | 0.81 | 0.09 | 0.19 | 0.98 | 0.80 | 0.65 |
| | GM | 0.90 | 0.42 | 0.33 | 1.36 | 1.40 | 1.10 |
| | Average... | 1.38 | 0.62 | 0.18 | 1.56 | 1.40 | 0.72 |
| | ±S.E.M.... | 0.21 | 0.18 | 0.03 | 0.19 | 0.19 | 0.08 |
| CF | CF2 | | | Fluid | | | |
| | CF4 | 1.32 | 0.65 | 0.22 | 1.54 | 1.41 | 0.75 |
| | CF1 | 2.00 | 1.17 | 0.15 | 2.08 | 1.85 | 0.60 |
| | JK | | | Fluid | | | |
| | JR | 1.80 | 1.08 | 0.19 | 2.02 | 1.83 | 0.65 |
| | CF3 | 1.53 | 0.71 | 0.15 | 1.62 | 1.27 | 0.46 |
| | Average... | 1.66 | 0.90 | 0.18 | 1.81 | 1.59 | 0.61 |
| | ±S.E.M.... | 0.15 | 0.13 | 0.02 | 0.14 | 0.15 | 0.06 |

as a result, $\tan\delta$ increased at higher frequencies. Thus whereas the viscous component of the gel only represented ~ 15 % of the elastic component of low frequency, this increased to ~ 40 % of the elastic component at high frequency.

Table 4 shows the rheological properties of gels reconstituted from purified mucins from eight N and six CF patients (H14 mucin was not analysed). Three mucins (one N and two CF) did not form gels even at the highest concentration studied in these experiments, namely 25 mg/ml. Wide variations were seen in the actual values of $G'$, $G^{\parallel}$ and $\tan\delta$ for individual mucins, but no statistical differences were detected between CF and N samples ($P > 0.1$). There was no correlation between the rheological properties of a mucin and either its protein content or the time *post mortem* that the intestinal tissue was originally obtained ($P > 0.1$). Similarly, no direct correlation was detected between rheological properties of a mucin and its S–S or SH content ($P > 0.1$).

## DISCUSSION

The objective of the present studies was to compare the S–S and SH content and the rheological properties of purified small-intestinal mucins from N subjects and patients with CF. To this end, we developed a simple but highly reproducible assay to measure S–S and SH groups using 4,4′-PDS, and critical stages of the assay procedure were optimized. Experimental values for the S–S and SH content of well-characterized proteins obtained using the standard assay agreed with expected values determined from structural and sequence data. Although consistent values were obtained for the S–S and SH contents of each of the 15 highly purified intestinal mucins (nine N and six CF) analysed in the present study, wide variations were observed in the S–S/SH contents of individual preparations, whether expressed relative to mucin dry weight, to the mucin protein content or per nmol of serine plus threonine in the mucin preparation. However, no statistical differences were detected between CF and N mucins. The reasons for the variability among mucin preparations is not yet known, although loss of mucin protein (particularly non-glycosylated peptide) by *post mortem* autolysis and/or degradation *in vitro* would seem to be a likely explanation. Assuming that, at the time of biosynthesis, the composition of the mucin peptide moiety is the same in different individuals, then the variability in S–S/SH content when expressed per mg of mucin protein or per nmol of serine plus threonine (which are largely located in proteinase-resistant glycosylated regions of the molecule) may reflect loss of protein domains from both N and CF mucins. However, variations in the S–S/SH contents of mucins did not parallel the variability in their protein content, i.e. those mucins with a high protein content did not necessarily have a high S–S/SH content (for example, CF1 and CF2). Also, neither the S–S/SH content nor the protein content of a mucin were directly related to the time *post mortem* that the intestinal tissue was obtained. Both these observations would argue against autolysis *post mortem* and loss of peptide being responsible for the variability in mucin S–S/SH content. Since all mucins were thoroughly purified in the presence of proteolytic inhibitors and were not exposed to reducing agents

during purification, and since at least 11 preparations contained only high-molecular-mass polymeric material, eluted in the excluded volume of a Sepharose-CL 2B column, it also seems unlikely that degradation *in vitro* was the cause for the variability in mucin S–S/SH and protein contents. As yet, therefore, we cannot readily attribute the inter-sample differences in S–S/SH content to loss of mucin protein, and further studies on the peptide core(s) of intestinal mucin are required before the variability in S–S/SH values may be explained.

From previous analyses of human intestinal mucin before and after proteolytic digestion it is known that most of the cysteine residues occur in non-(or poorly-glycosylated regions of the macromolecule, namely the disulphide-bound 118 kDa 'link' glycoprotein and the *C*-terminal segment of the peptide core of the mucin glycoprotein monomers (Mantle *et al.*, 1984*b*). If the 118 kDa glycoprotein served as a 'link' component holding together the mucin monomers by S–S bonds, one may expect to see a correlation between the amount of 118 kDa glycoprotein present in a mucin preparation and its content of S–S bonds. Previously we determined the 118 kDa glycoprotein content of the same CF and N mucins as those used in the present study (Mantle & Stewart, 1989). No correlation was detected between the S–S content of a mucin and its content of 118 kDa glycoprotein. This would seem to suggest that most of the S–S bonds may be located elsewhere in the mucin macromolecule, i.e. the 'naked' *C*-terminal segment of the peptide core of the mucin glycoprotein monomers. Since this peptide segment only comprises a very small proportion (< 10%) of the total mucin protein (Mantle *et al.*, 1984*b*), it is not surprising that we did not find a correlation between the overall protein content of a mucin and its S–S/SH content.

Rheological analyses of CF and N intestinal mucin gels showed that $G'$ was greater than $G''$ throughout the frequency range studied (1–100 rad/s) and that G′ was virtually independent of frequency. This pattern of behaviour, in conjunction with the relative magnitudes of $G'$ and $G''$, is characteristic of a weak but stable visco-elastic gel with cross-links that are not labile in the time course of the experiment. Both CF and N mucin gels maintained the same mechanical properties over a long period of time (14 days), presumably reflecting the purity of our mucin preparations and the absence of contamination by proteinases known to degrade and dissolve mucin gels (Bell *et al.*, 1985). The actual values of $G'$, $G''$ and $\tan\delta$ obtained for individual mucins, as well as the concentration at which mucins gelled, varied considerably among preparations. However, no systematic differences could be detected between N and CF mucins. The variability in rheological properties could not be related to the S–S content of the mucin, so the formation of a more 'rigid' gel (with a high $G'$, low $G''$ and low $\tan\delta$) could not be attributed to the presence of more S–S bonds in the mucin polymer. It was noted, however, that two (JK and CF2) of the three intestinal mucin preparations that did not gel in the present study had a relatively low content of S–S bonds and one (JK) contained Sepharose-CL 2B-partially-included (non-polymerized) material. Since cleavage of S–S bonds is known to dissociate the polymeric structure of mucin and destroy its gel-forming properties (Bell *et al.*, 1985), it is possible that a minimum number of S–S bonds are required to maintain the integrity of the mucin polymer and allow gel formation. Perhaps the low S–S content of JK and CF2 mucins was below that minimum and hence unable to support gel formation. Obviously, however, a greater understanding of mucin polymerization (the number of S–S bonds involved and where they are located) and gel formation is required before the inter-sample variability in S–S/SH content and visco-elastic properties can be fully explained.

The rheological properties of our human intestinal mucin gels (both N and CF) resemble those of unfractionated mucus gels and reconstituted, purified mucin gels from the pig stomach, small intestine and colon (Bell *et al.*, 1984; Sellers *et al.*, 1983, 1987, 1988) in showing little concentration-dependence. This behaviour is indicative of a gel with a relatively high sol fraction containing variable and even large amounts of mucin trapped within the interstices of the matrix. Accordingly, ~ 10% of the total mucin in a gel can be extracted (by leaching or gentle stirring) without affecting the rheological properties of the matrix (Bell *et al.*, 1984). Possibly, this phenomenon results from limited availability of water after the critical gel point such that further mucin molecules added to the gel cannot hydrate properly. Once the matrix has formed, it is so stable that trapped mucin molecules must be prevented from hydrating and contributing to the gel even in the presence of additional water, leading to the observation that mucus gels will not swell once formed. The complete lack of dependence between the visco-elastic properties of a mucin and its concentration above the gelation point is consistent with such a model.

The relative magnitudes of $G'$ ('solid-like' response) and $G''$ ('liquid-like' response), the value of $\tan\delta$ (the ratio of $G''/G'$), and the frequency-dependence of these parameters indicate the overall quality of a mucus or mucin gel. In pig gastric and colonic mucus and reconstituted mucin gels, $G'$ is substantially greater than $G''$, neither show appreciable frequency-dependence and $\tan\delta$ is < 0.2 over a wide range (0.1–100 rad/s) of frequencies (Sellers *et al.*, 1983, 1987, 1988). In human and canine tracheal (Jeanneret-Grosjean *et al.*, 1988; Khan *et al.*, 1976; King, 1980, 1981; Litt *et al.*, 1976) and human and bovine cervical mucus (Litt *et al.*, 1976), $G'$ does not predominate over $G''$ to the same extent, and $G''$ shows more frequency-dependence, particularly at frequencies above 10 rad/s. As a result, $\tan\delta$ varies between 0.3 and 1.0 over a frequency range of 0.1–100 rad/s, suggesting a greater tendency for these mucus gels to flow when compared with gastric and colonic mucus (King, 1987). The mechanical properties of our human intestinal mucin gels (both N and CF) were intermediate between those of the two groups above. $G'$ was greater than $G''$ and was largely unaffected by frequency, $G''$ showed a certain amount of frequency-dependence at higher frequencies, and $\tan\delta$ ranged between 0.2 and 0.7. At low to intermediate frequencies the gels were therefore similar to pig gastric and colonic mucus, with $\tan\delta < 0.2$, whereas at high frequency $\tan\delta$ rose to ~ 0.7 and the gels more closely resembled respiratory and cervical mucus. These findings are very similar to those observed previously in studies on the rheological properties of pig intestinal mucus and mucin gels (Sellers *et al.*, 1983, 1987). Thus it appears that small-intestinal mucins (as well as respiratory and cervical mucins) form somewhat poorer-quality gels than gastric and colonic mucins, although the reason(s) for this are not yet clear.

Pig gastric, intestinal and colonic mucus all have very similar rheological properties that may be reproduced *in vitro* by their purified constituent mucins (Bell *et al.*, 1984; Sellers *et al.*, 1983, 1987). A model for gel formation has been proposed which depends on the covalent polymeric mucins interacting non-covalently by relatively stable interdigitation of carbohydrate side chains from different molecules. More transient entanglements of these 'aggregates' lead to the formation of the gel matrix, which then incorporates large, variable, amounts of soluble mucin in its interstices (Bell *et al.*, 1984; Sellers *et al.*, 1988). Although the mechanism of gel formation appears to be the same in gastric, intestinal and colonic mucus, it is possible that the poorer quality of intestinal mucin gels is a reflection of the known differences between these mucins and gastric and colonic mucins in terms of their polymeric structures and carbohydrate side chains (Allen, 1981).

Contrary to most expectations, a number of previous studies showed that the viscosity of CF sputum was generally lower than that in other hypersecretory lung diseases such as chronic bronchitis and asthma (Charman & Reid, 1972; Feather & Russell, 1970; Lopez-Vidriero & Reid, 1978; Picot *et al.*, 1978). However, more complete analyses of the visco-elastic properties of CF sputum demonstrated no rheological abnormality except in the presence of infection (King, 1981). The present study also failed to detect a significant change in the overall gel-forming properties of purified intestinal mucin from CF patients, despite the fact that CF mucin is known to be more highly sulphated, have longer oligosaccharides (Wesley *et al.*, 1983*a*,*b*) and may have a different conformation than N intestinal mucin (Mantle & Stewart, 1989), all of which could potentially influence the rheological profile of CF mucin. Possibly, therefore, accumulation of mucus secretions in CF results from hypersecretion and/or diminished clearance. Changes in the chemical composition and physical conformation of CF mucin may alter its interactions with other components present in mucus *in vivo*, for example $Ca^{2+}$ and albumin (List *et al.*, 1978; Forstner & Forstner, 1976; Forstner *et al.*, 1977), leading to increased resistance of the mucin to the normal course of degradation and turnover. Examination of these issues will require more research.

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## REFERENCES

Allen, A. (1981) in Physiology of the Gastrointestinal Tract (Johnson, L. R., ed.), p. 617–639, Raven Press, New York
Bell, A. E., Allen, A., Morris, E. R. & Ross-Murphy, S. B. (1984) Int. J. Biol. Macromol. **6**, 309–315
Bell, A. E., Sellers, L. A., Allen, A., Cunliffe, W. J., Morris, E. R. & Ross-Murphy, S. B. (1985) Gastroenterology **88**, 269–280
Chaplin, M. F. (1982) Anal. Biochem. **123**, 336–341
Charman, J. & Reid, L. (1972) Biorheology **9**, 185–199
Fahim, R. E. F., Forstner, G. G. & Forstner, J. F. (1983) Biochem. J. **209**, 117–124
Feather, E. A. & Russell, G. (1970) Br. J. Dis. Chest **64**, 192–200
Forstner, J. F. & Forstner, G. G. (1976) Pediatr. Res. **10**, 609–613
Forstner, J. F., Jabbal, I., Findlay, B. P. & Forstner, G. G. (1977) Mod. Probl. Paediatr. **19**, 54–65
Forstner, J. F., Maxwell, B. & Roomi, N. (1981) Am. J. Physiol. **241**, G443–G450
Freye, H. B., Stanley, M. K., Spock, A. & Capp, M. P. (1964) J. Pediatr. (St. Louis) **64**, 575–579
Grassetti, D. R. & Murray, J. F. (1967) Arch. Biochem. Biophys. **119**, 41–49
Habeeb, A. F. S. A. (1972) Methods Enzymol. **25**, 457–464
Hinegardner, R. T. (1971) Anal. Biochem. **39**, 197–201
Jeanneret-Grosjean, A., King, M., Michoud, M. C., Liote, H. & Amyot, R. (1988) Am. Rev. Respir. Dis. **137**, 707–710
Jeffrey, I., Durrans, D., Wells, M. & Fox, H. (1983) J. Clin. Pathol. **36**, 1292–1297
Johansen, P. G. & Kay, R. (1969) J. Pathol. **99**, 299–306
Khan, M. A., Wolf, D. P. & Litt, M. (1976) Biochim. Biophys. Acta **444**, 369–373
King, M. (1980) Fed. Proc. Fed. Am. Soc. Exp. Biol. **39**, 3080–3085
King, M. (1981) Pediatr. Res. **15**, 120–122
King, M. (1987) Biorheology **24**, 589–597
King, M. (1988) in Methods in Bronchial Mucology (Braga, P. C. & Allegra, L., (eds.), pp. 73–83, Raven Press, New York
List, S. J., Findlay, B. P., Forstner, G. G. & Forstner, J. F. (1978) Biochem. J. **175**, 565–571
Litt, M., Khan, M. A. & Wolf, D. P. (1976) Biorheology **13**, 37–48
Lopez-Vidriero, M. & Reid, L. (1978) Am. Rev. Respir. Dis. **117**, 465–477
Mantle, M. & Forstner, J. F. (1986) Biochem. Cell Biol. **64**, 223–228
Mantle, M. & Stewart, G. (1989) Biochem. J. **259**, 243–253
Mantle, M. & Thakore, E. (1988) Biochem. Cell Biol. **66**, 1045–1054
Mantle, M., Mantle, D. & Allen, A. (1981) Biochem. J. **195**, 277–285
Mantle, M., Forstner, G. G. & Forstner, J. F. (1984*a*) Biochem. J. **217**, 159–167
Mantle, M., Forstner, G. G. & Forstner, J. F. (1984*b*) Biochem. J. **224**, 345–354
Morrissey, S. M. & Tymvios, M. C. (1978) J. Pathol. **126**, 197–208
Neutra, M. R. & Trier, J. S. (1978) Gastroenterology **75**, 701–710
Neutra, M. R., Grand, R. J. & Trier, J. S. (1977) Lab. Invest. **36**, 535–546
Park, C. M., Reid, P. E., Owen, D. A., Sanker, J. M. & Applegarth, D. A. (1987) Exp. Mol. Pathol. **47**, 1–12
Pearson, J. P., Allen, A. & Parry, S. (1981) Biochem. J. **197**, 155–162
Picot, R., Das, I. & Reid, L. (1978) Thorax **33**, 235–242
Riddles, P. W., Blakeley, R. L. & Zerner, B. (1983) Methods Enzymol. **91**, 49–60
Sellers, L. A., Allen, A., Morris, E. R. & Ross-Murphy, S. B. (1983) Biochem. Soc. Trans. **11**, 763–764
Sellers, L. A., Allen, A., Morris, E. R. & Ross-Murphy, S. B. (1987) Biorheology **24**, 615–623
Sellers, L. A., Allen, A., Morris, E. R. & Ross-Murphy, S. B. (1988) Carbohydr. Res. **178**, 92–110
Wesley, A. W., Forstner, J. F. & Forstner, G. G. (1983*a*) Carbohydr. Res. **115**, 151–163
Wesley, A. W., Forstner, J. F., Qureshi, R., Mantle, M. & Forstner, G. G. (1983*b*) Pediatr. Res. **17**, 65–69

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