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MUCUS GLYCOPROTEIN GELS. ROLE OF GLYCOPROTEIN POLYMERIC STRUCTURE AND CARBOHYDRATE SIDE-CHAINS IN GEL-FORMATION*

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ABSTRACT

The structure of mucus glycoprotein gels from the pig gastrointestinal tract was investigated by mechanical spectroscopy. Gastric, duodenal, and colonic mucus had the same mechanical profile, characteristic of a viscoelastic gel. The gel structure collapsed on destruction of the polymeric structure of the component glycoprotein by reduction with 0.2M mercaptoethanol or after proteolysis with papain. The progressive weakening of mechanical properties and the decrease in polymeric glycoprotein content were measured as functions of time of reduction. A linear correlation was obtained between the gel quality [defined by tan δ, the ratio of the loss modulus (G'') to the storage modulus (G')] and the proportion of polymeric to subunit glycoprotein in the mucus. Purified mucus glycoprotein, at the same concentration as that in native mucus, resulted in a gel with mechanical properties no different from those of the respective native secretion, demonstrating that the glycoprotein alone could reproduce the gel-forming properties of mucus. After proteolytic digestion, all native secretions and reconstituted mucus showed an absence of Newtonian behaviour in the frequency dependence of dynamic viscosity at low frequencies. This provided evidence that the noncovalent interactions, characteristic of the native gel matrix, were still present after proteolytic digestion when the nonglycosylated protein core accessible to proteinases had been removed. These results were interpreted to show (a) a common mechanism for gel-formation in gastric, duodenal, and colonic mucus; (b) that the polymeric structure of mucus glycoproteins confers the three-dimensional structure necessary for

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formation of the gel network; and (c) that noncovalent interactions which arise between the glycoprotein molecules by relatively stable interdigitation of the carbohydrate side-chains are involved in formation of the gel network.

INTRODUCTION

Gastrointestinal mucus occurs as a stable, water-insoluble gel adherent to the mucosal surface, together with a soluble form in the lumen[1]. The adherent mucus gel can be observed on unfixed sections of gastric mucosa as a translucent, continuous layer of median thickness 180 μm in man and 80 μm in rat[2]. The primary function of gastrointestinal mucus is to provide a protective barrier between the underlying epithelium and the hostile environment of the lumen. In stomach and duodenum, the adherent mucus, with an epithelial hydrogen carbonate secretion, is considered to be the first line in defence against mucosal ulceration by acid and pepsin from gastric juice[3,4]. Mucus also has a role in protection against gut pathogens[5], and in the intestines provides an essential microclimate at the epithelial surfaces for the endogenous bacterial flora[6,7]. A more general function of mucus throughout the gastrointestinal tract is to act as a lubricant to prevent epithelial damage from the mechanical forces of the digestive processes. An impairment of the gastric mucus barrier is associated with peptic ulcer disease[8], while the structure of the carbohydrate chains of colonic mucus glycoprotein is changed in ulcerative colitis[9,10]. For a full understanding of the roles of mucus in health and disease, it is necessary to elucidate the structure of mucus glycoprotein gels.

The structure of mucus glycoprotein subunits is well established as a protein core to which are attached many closely packed carbohydrate side-chains[11–13]. In pig gastric mucus, these carbohydrate side-chains are up to 19 sugars in length[14] and occur, on average, every 3–4 amino acid residues along the protein core but sometimes on adjacent residues[15]. The sheath of carbohydrate, 70–80% by weight of the glycoprotein, protects the protein core from proteolytic attack, but other peptide regions which are not glycosylated are susceptible to proteolysis[16,17]. In gastric mucus, ~50% of the protein core (or 6.7% by weight of the glycoprotein) is lost on proteolysis[18].

An important feature of mucus glycoproteins is their polymeric structure whereby glycoprotein subunits are joined together through disulphide bridges between nonglycosylated regions of the protein cores[13,19]. The size of the polymeric glycoprotein varies according to the isolation procedure used and source of mucus[20,21]. For example, preparations of pig gastric mucus glycoprotein isolated by different groups[20,22] ranged in size from M_r ~2 to ~45 10^6. Proteolysis or reduction of polymeric pig gastric mucus glycoprotein produced[20] glycoprotein subunits of M_r ~5 10^5. Polymeric colonic mucus glycoprotein (M_r ~15 10^6) yielded on proteolysis[23] glycoprotein units of M_r ~7.0 10^5. Similar polymeric structures exist in small intestinal[24,25] and duodenal mucus[26], as well as in respiratory[27,28] and cervical mucus[22,29], and, in all cases, the size of the proteolytically fragmented glycosylated

units is between M_r ~2 to 7 10^5. The solubilisation of mucus secretions by proteolysis or reduction has long been established[30,31], and elucidation of the polymeric structure of mucus glycoproteins explained these mucolytic actions in molecular terms.

Information about mucus gel structure can be obtained, without its disruption, from mechanical spectroscopy[32]. Small oscillatory deformations of fixed frequency (ω, over the range 10^{-2}–10^2 rad·s^{-1}) are applied to the gel sandwiched between a flat plate and shallow cone. From the magnitude of the stress generated by the mucus sample in resisting deformation and the phase relationship between this and the applied strain, the degrees of solid-like (storage or elastic modulus G') and liquid-like (loss or viscous modulus G'') properties of the sample are obtained. Rheological studies on mucus secretions have shown both respiratory[33–35] and gastric mucus[26] to be true viscoelastic gels, although the former has more tendency to flow. A model has been proposed for gastric mucus structure in which the functional interactions between the native polymeric glycoprotein molecules arise noncovalently, through interdigitation of the carbohydrate chains[36].

Herein, we report further the role of the polymeric structure and carbohydrate side-chains in formation of mucus glycoprotein gels. This, we have done by monitoring with mechanical spectroscopy the progressive breakdown of gel structure on reduction or proteolysis.

EXPERIMENTAL

Materials. — Pig gastric, duodenal, and colonic mucus gel samples were obtained by gently scraping the surface of the washed mucosa with a small plastic scoop avoiding, as far as possible, the removable of epithelial cells. The intestinal mucosal surfaces were exposed by longitudinal dissection, preventing any distention of the tissue. Mucus from a minimum of 15 stomachs, 25 duodenums, or 40 colons was pooled and either investigated immediately or stored at −20° until required. The glycoprotein content of the native gels was assayed by the modified periodic acid–Schiff (PAS) method[37] after the sample of gel had first been exhaustively digested with papain (EC 3.4.22.2) and dialysed against 0.2M NaCl–0.02% (w/v) NaN_3 to remove protein.

Reconstituted mucus gels were formed by concentrating the solution of the glycoprotein isolated from native gastric, duodenal, or colonic mucus. Pooled mucus scrapings, in 4 times their own volume of ice-cold 0.2M NaCl–0.02% (w/v) NaN_3, were homogenised in a Waring blender at full speed for 1 min at 4°. After centrifugation (6000*g*, 1 h), the supernatant was strained through glass wool to remove insoluble lipid-rich material. The glycoprotein was separated from protein and nucleic acid by equilibrium centrifugation in a CsCl density gradient[38]. Mucus glycoprotein fractions from the first gradient were further purified by subsequent centrifugation in a CsCl density gradient. After centrifugation, the glycoprotein fractions were pooled, dialysed against 0.2M NaCl–0.02% (w/v) NaN_3, con-

centrated by vacuum dialysis, and redialysed against 0.2M NaCl–0.02% (w/v) NaN_3. The isolated glycoprotein was concentrated to 46–50, 35–40, and 19–22 mg·mL^{-1} for gastric, duodenal, and colonic mucus, respectively, to give reconstituted gels with glycoprotein concentrations comparable to those of the native secretions. Glycoprotein samples used to prepare each batch of gel (400–500 μg of glycoprotein) were run on 7.5% poly(acrylamide) disc gels (7 cm long) in the presence of sodium dodecyl sulfate[39] and stained for protein with Coomassie Blue.

Methods. — Mechanical spectroscopy. Dynamic oscillatory measurements on native gel samples or gels reconstituted from isolated glycoproteins were performed on a Rheometrics Mechanical Spectrometer (Rheometrics, Piscataway, NJ, U.S.A.) using a cone and plate system (cone diameter 25 mm, cone angle 0.1 radian). Sinusoidal deformation of the sample under test (sample volume ~1.5 mL) was produced by an electronically controlled servo system (range 10^{-3}–10^{2} rad·s^{-1} with a resolution of better than 0.1% per decade) driving the cone. The energy transmitted to the lower plate was detected by a transducer (Rheometrics, type ST, 0.01–10 g·cm^{-1}), mounted on an air bearing to reduce frictional losses. Environmental control of the sample chamber was by forced air convection, the air being supplied from a thermostatted water bath and humidified at the working temperature of 25°. Sample temperature was regulated to ±0.30° and sensed by use of a Pt resistance thermometer. The storage modulus G' (elastic component), the loss modulus G'' (viscous component), and the dynamic viscosity η^* were expressed as functions of frequency (frequency range 10^{-2}–10^{2} rad·s^{-1}) at a constant strain of 20%.

After loading, all samples were allowed to equilibrate (15 min) to the measuring temperature and to relax from any major stresses that may have been induced by the loading procedure. The strain profiles did not change over a period of 2 h, showing that 15 min was long enough for stresses to relax. The mechanical properties of the mucus samples were also unchanged over a period of 2.5 h (four successive frequency scans), showing that dehydration of the gel or other mechanical damage was not a factor in the measurements. The mechanical properties of the native mucus gels stored at −20° have been shown to be identical to those obtained for fresh native samples, provided the gels were left to recover for 1.5 h or more after thawing[36]. Strain scans on all mucus gels showed that both the storage (G') and loss (G'') moduli were independent of strain in the range 5–95%, at a frequency of 10 rad·s^{-1}.

Reduction and proteolysis. The native gels or the gels reconstituted from isolated mucus glycoprotein were reduced by dialysis against 0.2M mercaptoethanol in 0.2M Na phosphate buffer at pH 8.0 [0.02% (w/v) NaN_3] at either 4 or 20°. Mucus samples were also reduced at 37° by incubation, with gentle agitation, of 1.4–1.5 mL of gel in four times this volume of reducing buffer (final concentration, 0.2M mercaptoethanol).

Proteolytic digests of the gel samples were obtained with pepsin (EC 3.4.23.1) (1 mg·mL final concentration) or papain (20 μg/mg of protein digested)

at 37°. The mucus gels (1.4–1.5 mL) were submerged in four times their volume of pepsin buffer (0.2M citric acid adjusted to pH 2.2 with 5.0M NaOH) and gently agitated for various time intervals. Digestion by papain was carried out with both the mucus and enzyme contained in a dialysis sac immersed in 0.1M KH_2PO_4–Na_2HPO_4 buffer at pH 6.5 containing 5mM cysteine and 5mM sodium ethylenediamine tetraacetate.

For all reduction and proteolytic digestion procedures, control samples of gel were incubated or dialysed over the same time-course under the aforementioned conditions but with omission of mercaptoethanol or proteinase. The mechanical spectra of these control samples did not change significantly during the procedures (<3% change in the absolute value of any parameter). After measurement of mechanical spectra, all gel samples were stored at −20° until analysed. The glycoprotein concentration was determined by the PAS method[37], after exhaustive digestion of the samples with papain and dialysis to remove protein. All mucus samples were also analysed for the presence of degraded glycoprotein by gel filtration on a Sepharose CL-2B column (Pharmacia Ltd., Central Milton Keynes, U.K.). The mucus gel samples were individually solubilised by brief homogenisation in 0.2M NaCl–0.02% (w/v) NaN_3 (4 vols.). The samples were centrifuged (1000*g*, 10 min) to remove cell debris, the supernatant from each sample was loaded onto a Sepharose CL-2B column (1.5 × 140 cm) and eluted in all cases with 0.2M NaCl–0.02% (w/v) NaN_3. Fractions (3 mL) were collected and analysed for glycoprotein[37] and protein[40]. The percentage of total material recovered was 84–93%. The amount of excluded (fractions 19–31) relative to included glycoprotein (fractions 32–70) was calculated. The small PAS peak in the total volume (fractions 71–85) was ignored, since this was totally accounted for by protein interference in the PAS assay, and glycoprotein purified by equilibrium centrifugation in CsCl did not contain this peak.

RESULTS AND DISCUSSION

Mechanical spectroscopy measurements were performed on the water-insoluble adherent mucus gel, lightly scraped off the mucosal surface of the stomach, duodenum, and colon from freshly slaughtered pigs. All mechanical spectra were typical of viscoelastic gels with the elastic (or storage) modulus (G') showing little frequency dependence and having a value greater than the viscous (or loss) modulus (G'') throughout the frequency range accessed (Fig. 1). This pattern of behaviour has been observed previously for these mucus gel secretions[26,36,41]. Treatment of all three mucus gel preparations with 0.2M mercaptoethanol for 24 h at 20° or with papain (1–1.2 mg of papain/mL of gel) for 24 h at 37° caused complete solubilisation of the gels, and a loss of their mechanical structures such that the viscous modulus (G'') was now dominant over the elastic modulus (G') for much of the frequency range, a pattern characteristic of a viscous liquid. A similar collapse of gel structure occurred within 4 h when gastric or duodenal

mucus was incubated with 0.2M mercaptoethanol at 37° (Fig. 1). Mechanical spectroscopy measurements were made on several preparations of mucus gel which had been incubated with 0.2M mercaptoethanol for times shorter than that required for complete collapse of gel structure. These partially reduced gels showed mechanical properties intermediate between those of native and fully reduced mucus gels; both moduli showed an increased dependence on frequency, and the elastic modulus (G') showed a decreased value as the time taken for reduction progressed (Fig. 1).

After measurement by mechanical spectroscopy, each mucus sample was dissolved by brief homogenisation and centrifuged to remove cell debris, a procedure shown to solubilise over 74% by weight of the mucus glycoprotein[42]. The solubilised mucus in the supernatant was fractionated by gel filtration on Sepharose 2B. All native mucus preparations showed a single, large glycoprotein peak eluting in the excluded volume (Fig. 2). The only other PAS-positive material was a small amount which could be attributed entirely to interference in the PAS assay from protein and eluted in the total volume. After complete reduction, the glycoprotein-positive peak was eluted into the included volume of the Sepharose 2B column, approximately midway between the exclusion and inclusion limits (Fig. 2). These results are in agreement with previous gel filtration studies on purified pig gastrointestinal mucus glycoproteins[24,26,37], showing a clear separation of polymeric glycoprotein (excluded) and subunit glycoprotein (included) by this method. Glycoprotein from partially reduced samples of mucus was eluted as a continuum from the excluded into the included volume of the column, indicating incomplete reduction of the glycoprotein (Fig. 2). All glycoprotein fractionations were performed on the same Sepharose 2B column (with constant volume of gel and flow

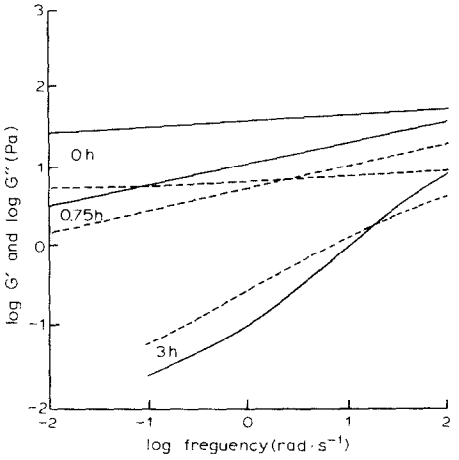

Fig. 1. Double logarithmic plot of the storage modulus (G', solid line) and loss modulus (G'', dotted line) against frequency (at 20% strain) for native pig gastric mucus gel incubated with 0.2M mercaptoethanol for 0, 0.75, or 3 h at 37°.

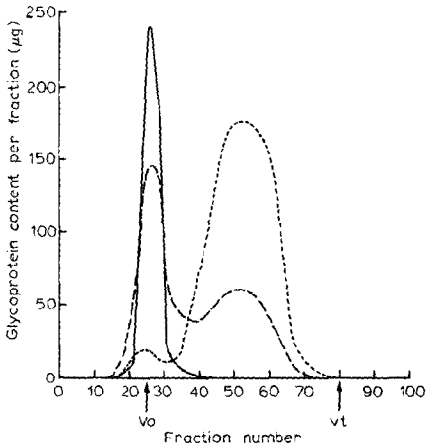

Fig. 2. Gel filtration analysis of reduced pig gastric mucus. Native pig gastric mucus gel was incubated for 0 (——), 0.75 (— —), or 3 (------) at 37° with 0.2M mercaptoethanol. The reduced sample was solubilised in 0.2M NaCl–0.02 (w/v) NaN_3 by brief homogenisation and the insoluble debris removed. The reduced, water-soluble mucus (total concentration 2–8 $mg \cdot mL^{-1}$) was applied (1.0 mL) to a Sepharose CL-2B column and eluted by upward flow with 0.2M NaCl–0.02% (w/v) NaN_3. Fractions (3.0 mL) of the column eluate were collected and analysed for glycoprotein (PAS).

rate) so that a comparison of the relative amounts of glycoprotein, either excluded (fractions 19–31) or included (fractions 32–70), in different reduced-mucus samples could be made. The proportion of the total mucus glycoprotein that was excluded from the column was seen to decrease progressively with increasing time of reduction. The time-course for this observed shift in size of the glycoprotein (from excluded to included material) is shown for gastric mucus in Fig. 3, and similar profiles were obtained for the glycoprotein from duodenal and colonic mucus on reduction.

It was clear that, with all three mucus secretions, gastric, duodenal, and colonic, the decrease in the amount of polymeric glycoprotein on reduction (as determined by gel filtration) was accompanied by a parallel collapse in the mechanical properties of the gel. To monitor quantitatively this progressive collapse of gel structure, the parameter tan δ was evaluated for each sample, at a frequency of 1 $rad \cdot s^{-1}$. Tan δ is the ratio of the loss modulus (G'') to the storage modulus (G') and gives a comparison of the "gel-like" and "liquid-like" behaviour of the mucus sample. Tan $\delta < 1$ indicates a predominantly elastic response (gel-like), whereas tan $\delta > 1$ indicates a predominantly viscous response (liquid-like). Thus, tan δ gives an overall indication of gel quality, and when plotted against the length of time for which the mucus sample has been exposed to the reducing agent, gives a useful method for quantifying the rate of collapse of gel structure on reduction. It was found that native gastric, duodenal, and colonic mucus gel secretions had tan δ values between 0.13 and 0.2, which progressively increased on reduction to >2.0, a value characteristic of a viscous liquid. The time-course of

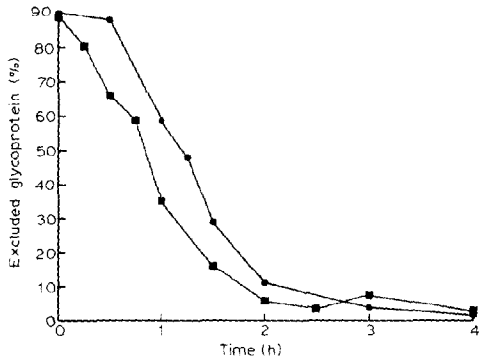

Fig. 3. The percentage of the total glycoprotein excluded on Sepharose CL-2B of native pig gastric mucus gels (■) or mucus gels reconstituted from isolated pig gastric mucus glycoprotein (●), after incubation with 0.2M mercaptoethanol at 37°, plotted against the respective time of reduction.

reduction for all three mucus secretions showed a close parallel between the progressive loss of gel quality (change in tan δ) and the decreased amounts of polymeric (excluded) glycoprotein in the same gel samples. Typical time-courses for the reduction of gastric mucus over a period of 4 h at 37° are shown in Figs. 3 and 4.

A direct comparison of the quality of the mucus gel preparations (expressed as tan δ) to the proportion of polymeric glycoprotein (excluded on Sepharose 2B) could be obtained by plotting the two parameters against each other. Such plots for gastric, duodenal, and colonic mucus sample at various stages of reduction are

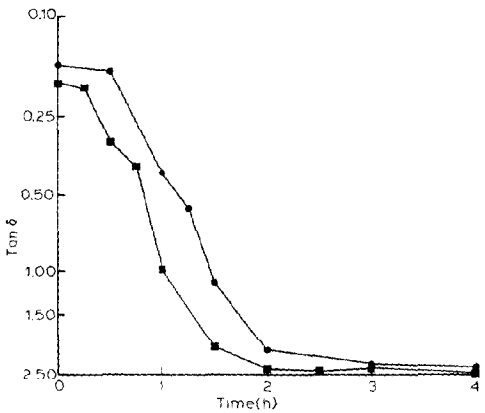

Fig. 4. Tan δ calculated at 1 rad·s^{-1} from the frequency profiles of native pig gastric mucus gels (■) or mucus gels reconstituted from isolated pig gastric mucus glycoprotein (●), after incubation with 0.2M mercaptoethanol at 37°, plotted against the respective time of reduction.

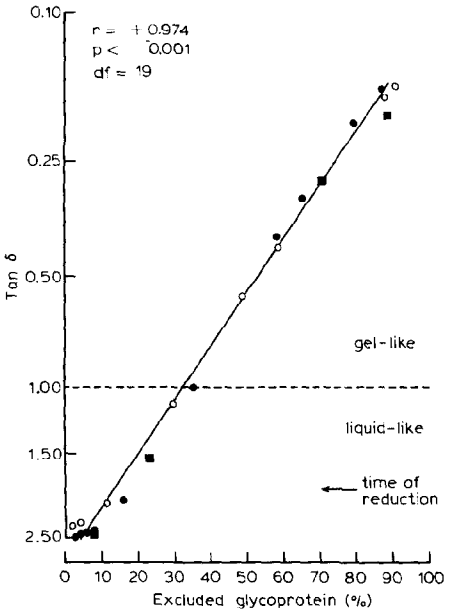

Fig. 5. Tan δ calculated at 1 rad·s^{-1} from the frequency profiles of native pig gastric mucus gels: (i) dialysed against 0.2M mercaptoethanol for 0, 6, 18, or 24 h at 4° (■), or (ii) incubated with 0.2M mercaptoethanol for 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, or 4 h at 37° (●), or (iii) mucus gels reconstituted from isolated pig gastric mucus glycoprotein incubated with 0.2M mercaptoethanol for 0, 0.5, 1, 1.25, 1.5, 2, 3, or 4 h at 37° (○), plotted against the percentage of the total glycoprotein excluded on Sepharose CL-2B. Regression analysis of the data in sets (i), (ii), and (iii) produced lines that, when compared by Cochran's modification to Student's T-test, were found to be identical except for a comparison of (i) with (ii) where the lines were parallel but separate (the probability of the gradients being the same was $p<0.208$ in all cases). Since the lines were of the same slope, the data were collapsed into a single figure.

shown in Figs. 5–7. Regression analysis of the data sets showed that they could all be treated as straight lines (lines correlated to within +0.964). Cross comparison of these lines by Cochran's modification to Student's T-test showed them not to be parallel ($p>0.001$ in all cases). Although this difference may reflect subtleties in structure, these were thought not to merit further interpretation since so many assumptions would be involved. However, what the plots did show was that, for all mucus secretions, there was a high correlation between tan δ and the proportion of polymeric glycoprotein.

Solubilised mucus glycoprotein (by use of mild shear) was purified by equilibrium centrifugation in a caesium chloride density gradient. Under these conditions, the glycoprotein (fractions 5–8) separated from the nucleic acid (A_{260}, fraction 9) as well as from the protein (fractions 1–3). Such purified gastric, duodenal, or colonic mucus glycoprotein was eluted from Sepharose 2B as a single peak in the excluded volume. Each glycoprotein preparation was free of non-covalently associated protein, as defined by the absence of any staining bands

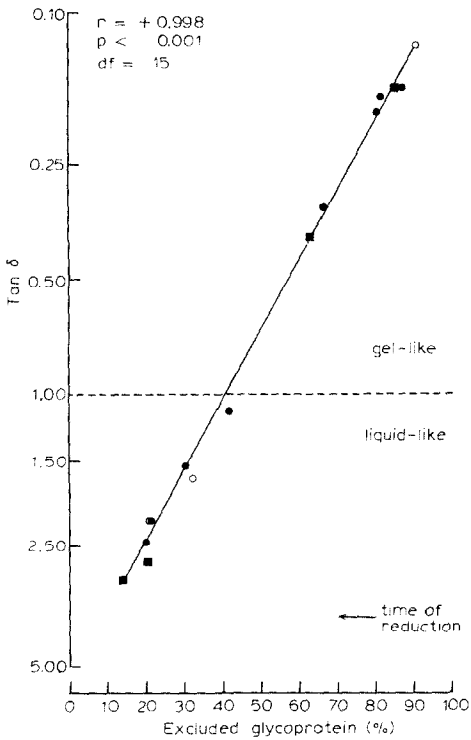

Fig. 6. Tan δ calculated at 1 rad·s^{-1} from the frequency profiles of native pig duodenal mucus gels: (i) dialysed against 0.2M mercaptoethanol for 0, 6, 18, or 24 h at 20° (■), or (ii) incubated with 0.2M mercaptoethanol for 0, 0.25, 0.5, 1, 1.5, 2, 3, or 4 h at 37° (●), or (iii) mucus gels reconstituted from isolated pig duodenal mucus glycoprotein dialysed against 0.2M mercaptoethanol for 0, 18, or 24 h at 20° (○), plotted against the percentage of the total glycoprotein excluded on Sepharose CL-2B. Regression analysis of the data sets (i), (ii), and (iii) produced lines that were parallel ($p<0.315$ in all cases for the gradient) when compared by Cochran's modification to Student's T-test. The data were collapsed into a single line.

(Coomassie Blue), after a high loading of glycoprotein (400–500 μg) was run on gel electrophoresis. The only band visible after electrophoresis was at the origin, where the glycoprotein had not entered the stacking gel. These glycoprotein preparations were concentrated by vacuum dialysis to the same concentration of glycoprotein as that present in the native mucus secretion, namely, gastric mucus ~50, duodenal mucus ~40, and colonic mucus ~20 mg·mL^{-1}. These reconstituted glycoprotein gels had the same form of mechanical spectrum as the corresponding native mucus secretion, characteristic of a viscoelastic gel. On reduction with 0.2M mercaptoethanol, the structure of the reconstituted gels collapsed, and the time-courses for the increase in tan δ and the decrease in polymeric glycoprotein content followed closely those of the corresponding native secretion (Figs. 3 and 4). Furthermore, plots of tan δ against the proportion of polymeric (excluded) glycoprotein were linear, and for reconstituted gastric and duodenal mucus the gradients of the lines

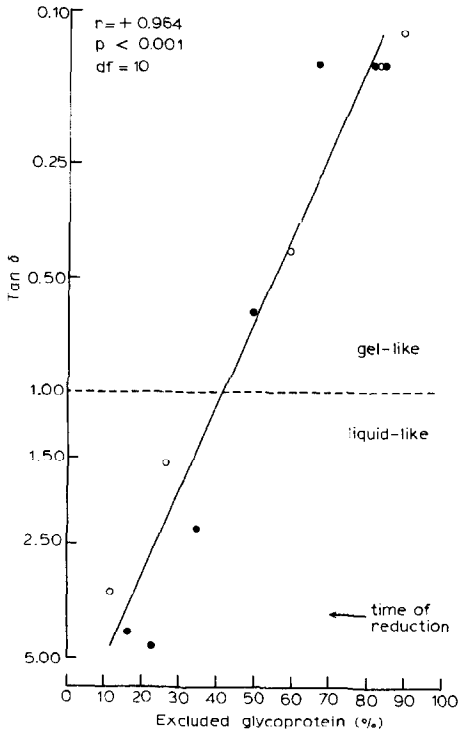

Fig. 7. Tan δ calculated at 1 rad·s^{-1} from the frequency profiles of native pig colonic mucus gels: (i) dialysed against 0.2M mercaptoethanol for 0, 3, 6, 12, 15, 18, or 24 h at 20° (●), or (ii) mucus gels reconstituted from isolated pig colonic mucus glycoprotein dialysed against 0.2M mercaptoethanol for 0, 6, 14, 18, or 24 h at 20° (○), plotted against the percentage of the total glycoprotein excluded on Sepharose CL-2B. Regression analysis of the data sets for native and reconstituted gels gave lines that were not parallel when compared by Cochran's modification to Student's T-test ($p>0.005$). The line illustrated is the best fit line through both data sets.

were identical ($p<0.495$) to those obtained for the respective native secretion, implying that the gels have very similar structures. In particular, all these reconstituted mucus gels showed the same dependence on the polymer structure of the glycoprotein for gel formation, as seen by the native mucus secretion. These studies showed that purified polymeric mucus glycoprotein, isolated by the method reported herein, will form a gel with mechanical properties the same as those of the native secretion. Therefore, with respect to the mucus samples in this study, there is no reason to suggest that other non-glycoprotein components play an integral part in gel-formation. Our studies showed these mucus glycoprotein preparations to be free of detectable protein and nucleic acid, whereas others have shown them essentially free of lipid[43,44].

Mucus gel samples, exhaustively digested with papain, had mechanical spectra typical of a viscous liquid, with G'' greater than G' over much of the frequency range accessed and a tan δ value of >2.4. On gel filtration, the digested

glycoproteins were eluted as single peaks from Sepharose 2B, in the same position as fully reduced glycoprotein (in all cases ~50% into the included volume). Progressive digestion of gastric, duodenal, or colonic mucus gel with papain or pepsin gradually decreased the size of the sample (from 1.5 mL) until the residual gel was too small (after ~2–4 h) to give measurable torque between the cone and plate of the spectrometer. Up until this point, the mechanical spectrum of the remaining undigested gel was similar to that of native mucus; on gel filtration the component glycoprotein from the undigested gel was eluted largely as a single excluded peak. These results are consistent with previous work on gastric and duodenal mucus, which have shown that proteolytic enzymes are too large to penetrate the interstices of the gel and that digestion proceeds by progressive solubilisation of the gel at its surface[26]. The success of the time-course experiments on reduction is due to the fact that, in contrast to proteinases, 0.2M mercaptoethanol can readily penetrate into the gel and thereby cause a progressive weakening of the total gel structure. Thus, a quantitative time-course for digestion of mucus samples proved impracticable.

The glycoprotein, isolated from the three pig gastrointestinal mucus secretions discussed here, has been shown to have a polymeric structure of subunits joined by disulphide bridges[13,19,26]. Extensive physical and chemical studies[13,17,20] on polymeric pig gastric mucus glycoprotein (M_r ~2 10^6) have shown it to consist of four subunits on average, of M_r ~5 10^5. These subunits were produced on reduction of the native glycoprotein. On proteolysis, degraded glycoprotein units of similar molecular size (M_r ~5 10^5) were produced which had lost the non-glycosylated protein core of the polymeric glycoprotein (~50% of the protein core: 6.7% by weight of the glycoprotein)[18]. A glycoprotein of substantially larger size (M_r ~45 10^6) has been isolated from pig gastric mucus in a mixture of proteolytic inhibitors and guanidinium hydrochloride[45]. At present, the size of the covalent, polymeric glycoprotein in native mucus gel is a matter of controversy, although temperature dissociation studies[46] indicated that the larger-sized glycoproteins are probably noncovalent aggregates of an entity of M_r ~2 10^6. The polymeric glycoprotein of M_r ~2 10^6 (isolated by the extraction method used in this study) is of consistent size, chemical analysis, and, in contrast to the larger-sized glycoproteins (M_r ~45 10^6), will form a gel having the mechanical properties of the native secretion[20,46]. After reduction or proteolysis, pig colonic mucus glycoprotein (M_r ~15 10^6) produced[23] a glycosylated glycoprotein fragment of M_r ~7 10^5. No molecular size studies have yet been performed on pig duodenal mucus, and the evidence for a polymeric structure is based on gel filtration profiles which are the same as those for other gastrointestinal glycoproteins where the polymeric structure is well established[26]. It should be noted that the conclusions in this paper do not depend on a knowledge of the true size of the polymeric glycoprotein, all forms of which are excluded on Sepharose 2B.

Gel filtration on Sepharose 2B provided a simple method for estimating the amount of reduced or proteolytically degraded glycoprotein of subunit size, in a sample of mucus. Whatever the size of the polymeric gastrointestinal glycoprotein

(*i.e.*, $M_r \sim 2\ 10^6$ or larger), it was excluded by Sepharose 2B and clearly separated from the completely reduced or proteolytically degraded glycoproteins in the included volume[20,21,24,26]. An example of such a separation for polymeric and reduced gastric mucus glycoprotein is seen in Fig. 2; this is typical of the gel filtration profiles obtained for gastric, duodenal, and colonic mucus glycoproteins after exhaustive reduction or proteolysis. The glycoprotein included in the gel will primarily be of subunit size ($M_r \sim 5\ 10^5$) without disulphide bridges joining subunits together. Some partially degraded glycoprotein, smaller than the native polymeric size but larger than the subunit, may also be eluted in the initial fractions of the included volume. A direct linear correlation was observed between the decreasing proportion of total glycoprotein excluded from Sepharose 2B, and the loss of gel quality, as defined by mechanical properties (Figs. 5–7). This correlation between polymer glycoprotein content and gel integrity was observed during the time-course of reduction for all three native mucus secretions (gastric, duodenal, and colonic) and for the gels reconstituted from the purified glycoproteins (Figs. 3–7). These results showed an absolute requirement for the polymeric glycoprotein in gel-formation, and that gel quality is proportional to the amount of glycoprotein present in the polymeric form. These studies determined quantitatively the dependence of gel formation on polymeric glycoprotein structure, an assumption previously made from viscosity studies on the isolated polymeric and reduced glycoprotein subunits[13,19]. It is of interest that a significant decrease in the proportion of polymeric mucus glycoprotein has been found in gastric mucus gel adherent to the antrum of gastric ulcer patients[8]. The amount of polymeric glycoprotein from gastric ulcer patients (mean 35% compared to a mean 67% in non-ulcer controls) was close to the cross-over point from the dominance of solid to dominance of liquid properties for the gel, as defined by tan δ (Fig. 5). This evidence supports the hypothesis that a physically weaker mucus barrier occurs in peptic ulcer disease.

After proteolytic cleavage, the glycoprotein might be expected to give a mechanical spectrum similar to that for a polymer solution, showing only simple entanglement[32], with $G'' > G'$ and, at low frequency, $G'' \alpha \omega$ and $G' \alpha \omega^2$. Such behaviour may also be described by use of the single parameter, η^*, the dynamic viscosity, defined by $\eta^* = [(G')^2 + (G'')^2]^{1/2}/\omega$. The frequency dependence of η^* follows very closely the shear-rate dependence of η, the usual steady shear viscosity. For entangled solutions, the measured viscosity at low frequency values is independent of increasing frequency (shear-rates) and shows a Newtonian plateau. For the same solution, at higher frequency values, the measured viscosity decreases with increasing frequency. Such behaviour is shown by hyaluronic acid, and also many other polysaccharide solutions[47]. Entanglement can be explained in simple terms by suggesting that, at low frequencies, topological intermolecular entanglements disrupted by flow are replaced by new interactions between different partners, with no net change in the extent of interaction. At higher shear-rates, the rate of removal of existing entanglements exceeds their rate of replacement, and the viscosity falls (shear thinning). To be able to compare the shear-rate

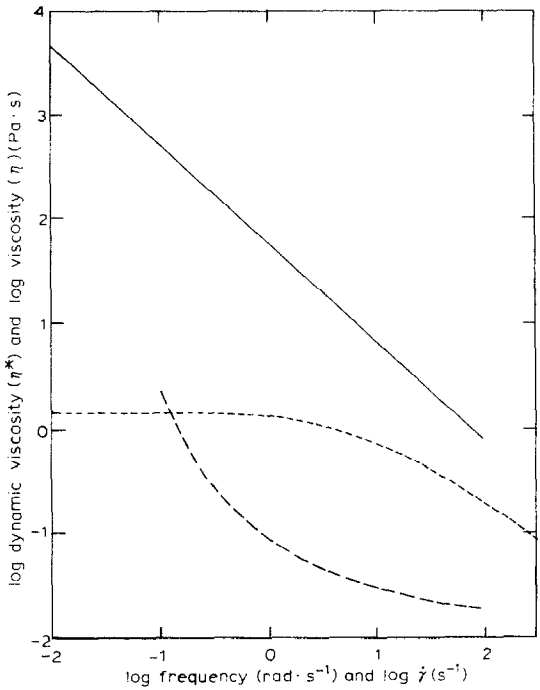

Fig. 8. Comparison of shear-rate ($\dot{\gamma}$) dependence of viscosity (η) for hyaluronic acid (------) with the frequency dependence at constant strain (20%) of dynamic viscosity (η^*) for: (i) native pig duodenal mucus (——), and (ii) native pig duodenal mucus digested with papain for 24 h at 37° (— —). The intrinsic viscosity of the hyaluronic acid sample was 2.6 mL·mg^{-1}, giving a $c[\eta]$ value of 14.3 at a concentration of 5.5 mg·mL^{-1}. For the sample of native pig duodenal mucus, $c[\eta]$ = 5.6 (35.2 mg·mL^{-1}·0.16 mL·mg^{-1}), and for the sample digested with papain $c[\eta]$ = 1.2 (36.2 mg·mL^{-1}·0.033 mL·mg^{-1}). Similar profiles were obtained for native pig gastric mucus gel and proteolytically degraded gastric mucus with coil overlap values of 13.8 and 3.8, respectively.

dependence of hyaluronic acid and mucus gels, the degree of volume occupancy of the polymeric species must be comparable. This may be characterised by a coil-overlap parameter given by $c[\eta]$, (concentration × intrinsic viscosity). The values for coil overlap used for the mucus samples are given in Figs. 8 and 9, and in all cases are comparable to or less than that for hyaluronic acid (14.3). Clearly for both the native mucus gels and the proteolytically digested mucus glycoprotein solutions, extra intermolecular interactions in addition to simple entanglement were observed, and are discussed below.

Native mucus gels from the stomach, duodenum, and colon, in contrast to hyaluronic acid (but all within the same coil overlap range), showed no evidence for a shear-independent plateau over a comparable shear-rate range (Figs. 8 and 9). This showed that the time-scale of intermolecular associations within the intact mucus gels is longer than would be anticipated for a purely entangled system, and compatible with the insoluble, gelatinous nature of the secretion. What is particularly interesting is that no shear-independent Newtonian plateau was seen

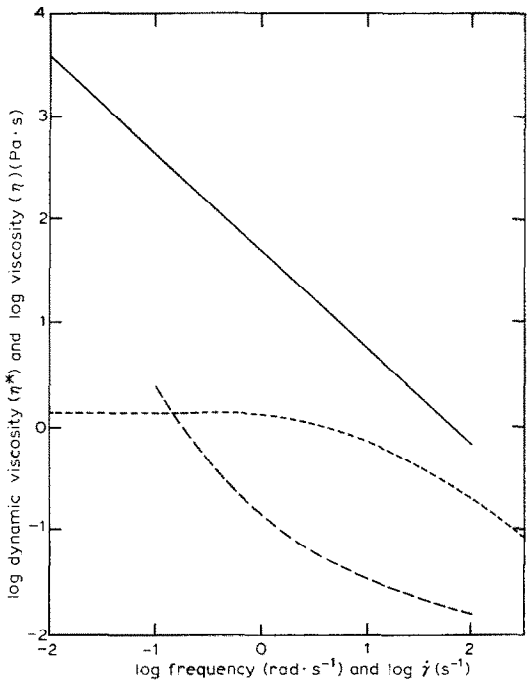

Fig. 9. Comparison of shear-rate ($\dot{\gamma}$) dependence of viscosity (η) for hyaluronic acid (------) with the frequency dependence at constant strain (20%) of dynamic viscosity (η^*) for: (i) native pig colonic mucus (——), and (ii) native pig colonic mucus digested with papain for 24 h at 37° (— —). The intrinsic viscosity of the hyaluronic acid sample was 2.6 mL·mg^{-1}, giving a $C[\eta]$ value of 14.3 at a concentration of 5.5 mg·mL^{-1}. For the sample of native pig colonic mucus, $c[\eta]$ = 6.7 (19.1 mg·mL^{-1} × 0.35 mL·mg^{-1}), and for the sample digested with papain, $c[\eta]$ = 1.6 (23.6 mg·mL^{-1} × 0.069 mL·mg^{-1}).

when the dynamic viscosity of mucus glycoprotein, after exhaustive proteolytic digestion (Figs. 8 and 9) or after reduction with mercaptoethanol, was plotted against frequency. This showed that, in addition to entanglement between degraded glycoprotein components, there is also appreciable intermolecular association leading to the formation of larger aggregates. The reasonable deduction is that noncovalent, intermolecular interactions involved in gel formation are retained by the proteolytically-degraded or reduced glycoprotein fragments after breakdown of the polymeric structure. The slightly concave appearance of the dynamic viscosity profiles for the degraded glycoprotein, not seen with the intact mucus gel, may reflect the mechanical breakdown of these aggregates. Some data on this has previously been reported for pig gastric mucus[36], but that for duodenal and colonic mucus (Figs. 8 and 9) are novel.

The nature of the noncovalent, gel-forming interactions between the polymeric glycoprotein molecules is unknown, but from the aforedescribed mechanical spectroscopy evidence, they are clearly more permanent than those resulting from physical entanglement. The fact that the same interactions are

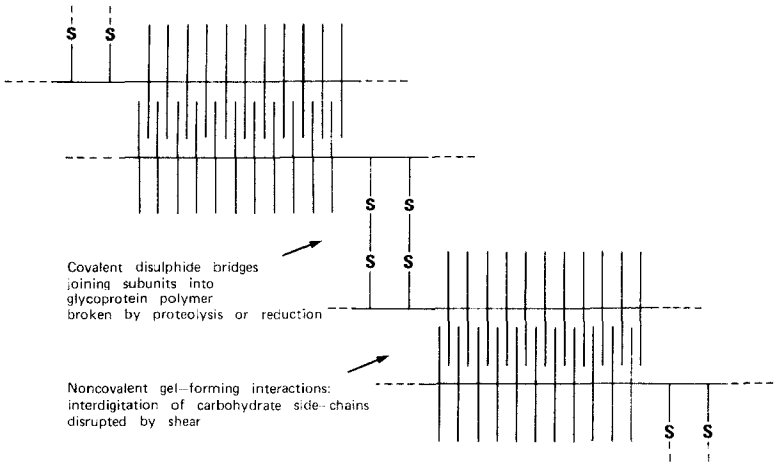

Fig. 10. A diagrammatic representation of proposed cross-links in mucus gel-formation.

observed between the glycoprotein components after exhaustive proteolytic digestion, where all the protein core accessible to proteolysis has been lost[18,23,24], is good evidence to suggest that these presumed gel-forming interactions are between the carbohydrate side-chains. The denaturants, 2.0M sodium chloride, 6.0M guanidinium chloride, and 8.0M urea, do not disrupt the gel structure and this, together with the ability of both native and reconstituted gels to spread and anneal given sufficient time[36], argues against co-operative interactions of ordered structures such as occur in some polysaccharide polymers[48]. This lack of effect of known protein denaturants on the gel also supports the proposal that it is the carbohydrate rather than the protein component that is involved in these intermolecular interactions. A model for such interactions is that, with increasing polymer concentration, the branched carbohydrate side-chains of adjacent molecules are forced to interdigitate (Fig. 10), giving rise to relatively stable and long-lived intermolecular associations, largely (though not exclusively) topological in nature. The existence of interactions between the subunits of mucus glycoprotein also supports the deduction that a decrease in size (rather than a loss of interaction) is the primary cause for the loss of mechanical properties of mucus on breakdown of the polymeric structure by reduction or proteolysis.

This work shows that two major structural features of mucus glycoproteins are essential for gel-formation (Fig. 10): (a) The covalent polymeric structure of the component glycoprotein confers the necessary three-dimensional arrangement for formation of the gel network, and (b) the noncovalent interactions between the glycoprotein molecules to form the gel network arise by a relatively stable interdigitation of the carbohydrate side-chains. These carbohydrate side-chains will confer hydrophilic characteristics to the molecule. Further, the charge and close packing of the carbohydrate side-chains will result in a stiffening of the glycoprotein molecule which may decrease the entropy change associated with gel-formation.

This model for gel structure applies to mucus secretions from the stomach, duodenum, and colon, and may well be a general model for mucus secretions from other body tracts. Although these interactions could account for gel formation, the data do not exclude the presence of other noncovalent interactions between the polymeric glycoprotein molecules to form the gel matrix. Further studies are necessary to show what other noncovalent interactions and precise structural features of the carbohydrate chains and polymeric structures of the glycoproteins are essential to mucus gel-formation.

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