Biochem. J. (1978) **175**, 565–571
Printed in Great Britain

Enhancement of the Viscosity of Mucin by Serum Albumin

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(*Received* 16 *March* 1978)

The interaction of serum albumin with a model epithelial mucin from pig stomach was explored by rotary viscometry. During 30min of incubation of human serum albumin (20mg/ml) and pig gastric mucin (8mg/ml) in iso-osmotic buffers at 37°C, the solution became markedly viscous. Viscosity enhancement was proportional to albumin concentration (2–40mg/ml), was most pronounced under conditions of low shear rate ($<45 s^{-1}$), and was considerably greater than the additive or multiplicative viscosity values calculated from albumin or mucin solutions measured separately. The viscous mucin–albumin complex was destroyed by high shear rates ($>90 s^{-1}$), but slowly re-formed under zero shear conditions. Elevation of pH (7 to 9), ionic strength (0.1 to 1.0), and addition of disodium EDTA (5mM) did not cause marked or specific alterations in the viscosity of the mixture, suggesting that electrostatic interactions probably do not stabilize mucin–albumin complexes. Urea (7M) and heating (35 to 55°C) caused a major increase in the viscosity of mucin and mucin–albumin mixtures, suggesting that rupture of hydrogen bonds, unfolding and partial denaturation of mucin promotes greater intertangling (possibly hydrophobic interactions) between mucin and albumin molecules. The implications of mucin–albumin interaction in diseases associated with mucus obstruction are briefly discussed.

Gastrointestinal mucus of mammals and other vertebrates is a viscous secretion lining the intestinal tract, and composed chiefly of high-molecular-weight mucin glycoproteins (Allen *et al.*, 1974; Forstner *et al.*, 1973*a*,*b*; Jabbal *et al.*, 1976; Pigman & Moschera, 1973). Until recently it has been assumed that mucus is an inert blanket, serving as a mechanical barrier against potentially injurious chemicals, bacteria and enzymes (Florey, 1962).

In recent years, however, it has become apparent that mucin glycoproteins are capable of interacting in various ways with many biologically important compounds, such as enzymes (Hochstraber *et al.*, 1972; Shora *et al.*, 1975; Hao *et al.*, 1977), cations (Bella & Kim, 1973; Deman *et al.*, 1973; Forstner & Forstner, 1975; Simmonds & James, 1976), drugs (Sheffner, 1963; Davis *et al.*, 1975; Marriott & Kellaway, 1975), viruses (DiGirolamo *et al.*, 1977), cell surfaces (Allen & Megan-Minnikin, 1975) and bacteria (Kashket & Donaldson, 1972; Hoskins & Boulding, 1976*a*, *b*). Some of these interactions alter mucus structure (Hoskins & Boulding, 1976*a*,*b*), solubility (Forstner & Forstner, 1976), permeability (Gibson *et al.*, 1971; Lukie, 1977; Marriott & Kellaway, 1975) and influence the protective functions of mucus (Strombeck & Harrold, 1974).

Interactions of mucins with other proteins have not been investigated systematically. Such interactions, if they exist, could affect the properties of mucus in both physiological and pathological states, since it is known that the quantity of non-mucin proteins within mucus is subject to considerable variation. For example, the non-mucin protein content of cervical mucus varies during the oestrous cycle, and it has been suggested that the hormone-associated changes in the consistency of cervical mucus may rely on local secretion of proteins (Elstein, 1970; Masson, 1973; Chantler & Debruyne, 1977). Mucus obtained from the respiratory, reproductive and intestinal tracts of patients with cystic fibrosis is characterized by its thick, viscous and sticky quality. The mucus is also highly enriched in protein, particularly albumin (Potter *et al.*, 1963; Schachter & Dixon, 1965; Di Sant'Agnese & Talamo, 1967; Knauff & Adams, 1968; Havez *et al.*, 1969; Dulfano & Adler, 1975). Roberts (1974, 1976) and Creeth *et al.* (1977) have shown that bronchial sputum glycoproteins are intimately associated with non-covalently bound proteins, suggesting that some positive interaction between these molecules may be taking place.

In view of the importance of possible protein–mucin interactions in causing qualitative changes in mucus consistency, we have investigated the influence of human serum albumin on the viscosity of a model mucin (Davis *et al.*, 1975; Deman *et al.*, 1973; Sheffner, 1963) derived from pig gastric tissue. Incubations of the two compounds were carried out

under conditions designed to mimick conditions *in vivo*, and an effort was made to elucidate the nature of the interaction.

Experimental

Preparation of mucin

(*a*) Pig gastric mucin (Sigma Chemical Co., St. Louis, MO, U.S.A.) was suspended in glass-distilled water (40mg of mucin/ml), containing 0.02% (w/v) NaN_3, and stirred overnight at 4°C. The suspension was centrifuged at 27000*g* for 15min and the supernatant removed. A second centrifugation (27000*g* for 15min) was carried out on the supernatant, followed by dialysis of the final supernatant against four to six changes of water. Dialysis resulted in the loss of 70% of the total protein, presumably owing to the removal of small peptide contaminants (< 10000mol.wt.). One further centrifugation (27000*g* for 15min) was carried out and the final supernatant stored at −20°C. The mucin produced by these steps is a soluble high-molecular-weight (> 1×10^6) glycoprotein containing 16–18% protein (per dry weight), and the sugars fucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine in molar proportions 0.43:1.96:1.63:1.0. No mannose or 2-deoxyribose could be detected, indicating no significant contamination of mucin preparations by serum glycoproteins or DNA.

Analysis of mucin sugars was carried out by g.l.c. by the methods of Clamp *et al.* (1971) and Chambers & Clamp (1971). Samples were treated with methanolic 1M-HCl at 100°C for 16h, neutralized with Ag_2CO_3 and acetylated with acetic anhydride. After centrifugation, the supernatants were dried and silylated with Tri Sil reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) in the presence of 17% (w/v) dimethyl sulphoxide. Silyl derivatives were extracted with hexane, concentrated and applied to 3% SE-30 on Chromasorb WHP 80–100 mesh columns (Chromatographic Specialities, Brockville, Ont., Canada) in a Varian aerograph GLC series 2100. Neutral sugars were determined during isothermal (160°C) runs, and sialic acid and amino sugars in a program of 160–230°C. Mannitol served as an internal standard. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard. The presence of DNA in mucin preparations (8mg/ml) was tested by the method of Schmid *et al.* (1963), which is capable of detecting as low as 2μg of 2-deoxyribose/ml. Mucin preparations contained less than 0.025% DNA by weight.

Incubations of pig mucin and serum albumin

These were carried out for 1h at 37°C in a Dubnoff metabolic shaking water bath at 30 cycles/min. Usually incubations contained 8mg of mucin (by weight) and 20mg of human serum albumin/ml in 0.1M-Tris/HCl or 0.1M-K_2HPO_4/KH_2PO_4 buffer, pH7.4. In the course of the investigation, conditions of mucin and albumin concentration, pH, buffer, temperature, time and shaking rate were altered, and details are specified in the Results section. In all experiments duplicate incubations were carried out for each variable tested, and the experiments were repeated at least twice thereafter on different days.

Proteins

Human serum albumin was obtained from Hoechst Pharmaceuticals (Willowdale, Ont., Canada). Fatty acid-free human serum albumin, bovine serum albumin and human haemoglobin were obtained from Sigma, and α-casein was from ICN Biochemicals (Cleveland, OH, U.S.A.). Altogether, three different batches of human albumin were tested. Purified rat tail collagen was donated by Dr. F. Keeley, The Hospital for Sick Children, Toronto, Canada. Purified human immunoglobulin G and its light chains were donated by Dr. B. Underdown, Clinical Sciences Division, University of Toronto.

Reagents

All buffer solutions were prepared in doubly distilled water from the most pure reagent-grade chemicals from either Sigma or Fisher Chemical Co. (Pittsburgh, PA, U.S.A.). Disodium EDTA was also purchased from Sigma.

Viscosity determinations

These were carried out in a Brookfield–Wells synchro-lectric rotary viscometer, model LVT with a 0.8° cone and a constant-temperature bath with an accuracy of ±0.0.1°C. Sample volumes were 1.5ml and were left for not less than 5min in the viscometer bath before viscosity readings were taken. Shear rates varied from 4.50 to 450s^{-1}. Viscosity was determined three times at each shear rate to ensure reproducibility and reliability of data. Differences in readings were less than 0.1% at shear rates of 11.25s^{-1} and higher. In each experiment viscosity readings were made of solutions of the solvent (buffers), solvent plus mucin, solvent plus albumin, and test mixtures containing all three. This enabled a calculation of specific viscosity ($\eta_{sp.}$) to be made in each case.

Results

Viscosity of albumin and mucin

To establish the best conditions for examining the effect of albumin on the viscosity of pig gastric mucin, viscosity experiments were first carried out

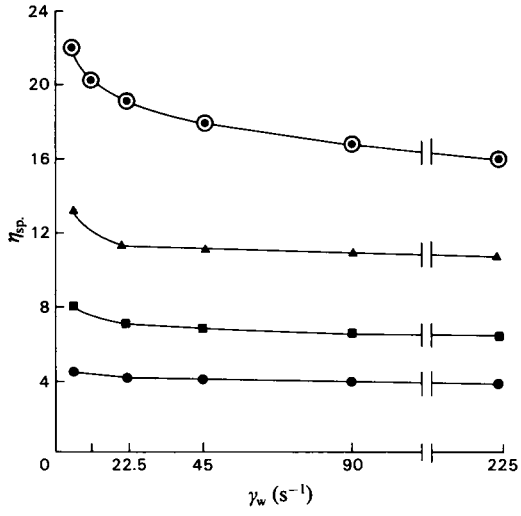

Fig. 1. *Viscosity of pig gastric mucin*
Mucin (4, 8, 12, 20mg/ml) (●, ■, ▲, ⊙) was incubated in 0.1M-Tris/HCl, pH7.4 at 37°C, in a slowly shaking (30 cycles/min) water bath for 1h. Viscosity was determined in a Brookfield–Wells rotary viscometer at 37°C. γ_w is shear rate (s^{-1}) and $\eta_{sp.}$ is specific viscosity.

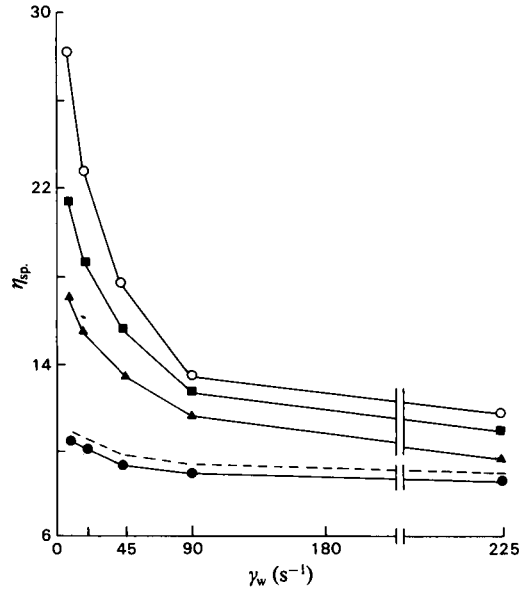

Fig. 2. *Effect of serum albumin on the viscosity of mucin*
Pig mucin (●) (8mg/ml) plus human (or bovine) serum albumin, at a concentration of 5(▲), 10(■) or 20(○) mg/ml, was incubated in iso-osmotic buffers for 1h at 37°C, and viscosities were measured. Albumin incubations (controls) were also performed, and the theoretical additive viscosity of mucin plus albumin (5–20mg/ml) is shown by the broken line. The data are from a representative experiment from a total of six that showed identical trends.

over a range of shear rates and concentrations. Solutions of human or bovine serum albumin (2–40mg/ml) in 0.1M-Tris/HCl, pH7.4, at 37°C, had specific viscosity values of 0.020–0.054 and showed no consistent variation with changes in concentration or shear rate. In contrast, mucin solutions varied significantly with concentration, and, at the higher concentrations, viscosity showed a definite increase with decreasing shear (Fig. 1). These trends are characteristic of mucins (Gibbons *et al.*, 1970; Gibbons, 1972; Pigman & Moschera, 1973) and are believed to be due to the intertangling of extended mucin threads at low shear, a process that eventually leads to gel formation. The tendency is less marked in the mucin preparation used for this study than in more 'native' gastric mucin preparations (Allen *et al.*, 1974), in which gentler conditions of isolation are used.

In further experiments we chose to use mucin concentrations of about 8mg/ml, ensuring high and reproducible viscosity readings at each shear rate, without marked shear-dependence. In each experiment, solutions of mucin, albumin and mucin–albumin mixtures were separately examined under identical conditions of concentration, temperature, incubation time, shear rates and added reagents, to compare their viscosity profiles.

The effect of incubating albumin with mucin at 37°C in iso-osmotic buffers is shown in Fig. 2. There was a dramatic enhancement of mucin viscosity in albumin-containing mixtures, which could not be explained by a simple additive effect of mucin viscosity plus albumin viscosity (broken line). The enhancement was proportional to the concentration of albumin, and was most marked at low shear rates. Replacement of the buffer by 0.01M-Tris/HCl/0.14M-NaCl, 0.1M-K_2HPO_4/KH_2PO_4 or 0.05M-K_2HPO_4/0.145M-NaCl, did not affect the results. Human serum albumin with or without bound fatty acids, and bovine serum albumin, gave identical results, whereas rat tail collagen (1–10mg/ml), human haemoglobin, human immunoglobulin G and bovine α-casein (2–20mg/ml) were without effect on mucin viscosity. Maximum enhancement of viscosity occurred after 30min of incubation with albumin in a 37°C shaking water bath at 30 cycles/min (not shown), and then showed no variation over the next 3h.

These findings suggested that a slowly forming viscous complex of albumin and mucin developed as the two were allowed to interact in an iso-osmotic

buffer at physiological pH and temperature. The observed shear-dependence suggests that formation of the complex relies on the ability of mucin fibres to intertangle with each other and with albumin. With increasing shear, the flexible fibres are forced to align themselves in the direction of flow and the viscosity decreases.

A test of the reversibility of shear-dependence was undertaken by subjecting albumin–mucin incubations to gradually increasing, then decreasing, shear rates, with periods of 5–15min between readings. Fig. 3 illustrates the effect on mucin alone and on mucin–albumin mixtures. A fully reversible change in viscosity was observed with mucin alone, but with mucin–albumin mixtures the original high viscosity was not fully regained within 15min. However, after several hours (12h standing at 4°C, followed by 20min at 37°C) the viscosity was measured again, and the original high viscosity had returned (broken line). The minimum time conditions necessary for full restoration of viscosity were not explored in this study. Nevertheless, the data show clearly that the viscosity effect of albumin on mucin is temporarily abolished by high shear rates, but is restored if given sufficient time to re-form with low-shear-rate conditions.

Test of ionic bonding between albumin and mucin

Incubation of mucin solutions in buffers ranging from pH7.0 to 9.0 was carried out in an effort to promote ionization of groups in the macromolecules with pK values near or within this range (e.g. imidazole, thiol, some free amino residues). Mucin viscosity fell an average of 25% as a result of this treatment (Table 1), suggesting that electrostatic interactions within the macromolecule normally help to stabilize its shape at pH7.4. Only minor decreases in the viscosity of albumin or mucin–albumin mixtures were noted, however, as pH was increased. Therefore ionic interactions involving protonated residues at or near pH7–9 do not appear to play a major role in stabilizing the viscosity of mucin–albumin mixtures.

Variations in ionic strength were also explored to assess their effects on the mucin–albumin interaction. One might expect that buffer ions would compete with intra- or inter-molecular electrostatic or ionic bonding (Kauzmann, 1959). Salt may also cause disruption and displacement of water molecules within the hydration sphere of a large polysaccharide-containing aggregate, and so decrease viscosity (Allen *et al.*, 1974). Experimentally, however, we observed very little change in mucin viscosity up to an ionic strength of 0.6, whereupon viscosity then decreased by 16% between 0.6 and 1.0 ionic strength (Fig. 4). With mucin–albumin mixtures, there was also little change until after 0.6, and then a decrease of 18.7% between ionic strength 0.6 and 1.0. Neither of these changes was very dramatic, and it seems unlikely that the extra salt ions interfered in a specific or major way with the interactions or shape of mucin–albumin complexes.

Since some mucins have been shown to bind Ca^{2+} (Forstner & Forstner, 1975) and other bivalent cations (Bella & Kim, 1973), and since albumin also binds bivalent cations (Perkins, 1961), we speculated

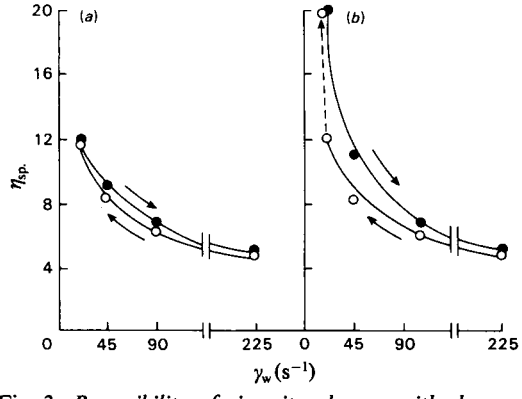

Fig. 3. *Reversibility of viscosity changes with shear rate* Mucin (*a*) (8mg/ml) or mucin–albumin mixtures (*b*) (8mg/ml and 20mg/ml respectively) were incubated in 0.1M-Tris/HCl, pH7.4, for 1h at 37°C. At 5 to 15min intervals thereafter the shear rate was increased in increments up to 225s^{-1} (●) and then decreased back to 22.5s^{-1} (○). Readings were taken at each increment. After regaining a shear rate of 22.5s^{-1}, the mucin–albumin sample was left at 4°C for 12h, rewarmed to 37°C for 20min, and the viscosity was remeasured (----).

Table 1. *Effect of pH on viscosity of mucin and mucin–albumin mixtures*

Samples of mucin, albumin or mucin–albumin mixtures were dialysed overnight at 4°C against 0.1M-Tris/HCl or 0.1M-phosphate buffers, pH7.5 or 9.0. After dialysis the solutions were adjusted to 37°C and the pH was checked. Incubations were then carried out for 1h at 37°C, and the pH and viscosity determined. Values of specific viscosity ($\eta_{sp.}$) are given for a representative experiment (shear rate 22.5s^{-2}). Identical trends were observed in two other experiments, although absolute viscosity values varied slightly between mucin preparations.

Sample	$\eta_{sp.}$ pH7.5	$\eta_{sp.}$ pH9.0	$\Delta\eta_{sp.}$ (%)
Mucin (9mg/ml)	8.3	6.2	25
Mucin (9mg/ml) + albumin (20mg/ml)	12.6	11.3	10
Albumin (20mg/ml)	0.0300	0.0286	4.67

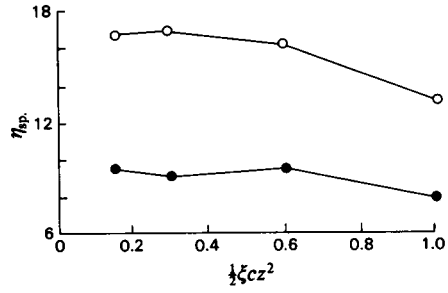

Fig. 4. *Effect of ionic strength on the viscosity of mucin–albumin mixtures*
Incubations of mucin (●) (8mg/ml) and mucin–albumin mixtures (○) (8mg/ml and 20mg/ml) were carried out at 37°C for 1h at pH7.4 in 0.05M-Tris/HCl buffer containing sufficient NaCl to give the desired ionic strength values ($\frac{1}{2}\xi cz^2$, where c is concentration and z is charge). The values presented were obtained at a shear rate of $45s^{-1}$.

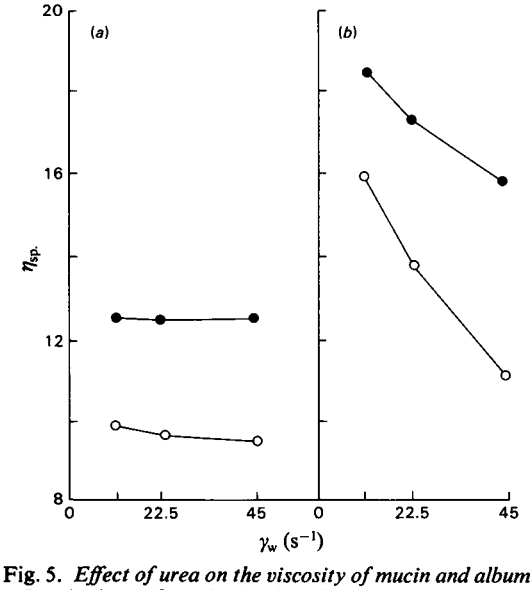

Fig. 5. *Effect of urea on the viscosity of mucin and albumin*
Incubations of mucin (*a*) (8mg/ml), and a mixture of mucin plus albumin (*b*) (20mg/ml) were carried out at 37°C for 1h in 0.1M-Tris/HCl, pH7.4, with (●) or without (○) a final concentration of 7M-urea.

that mucin–albumin interactions might involve Ca^{2+} 'bridging' of anionic groups on each molecule. Incubations containing disodium EDTA (up to 5mM), however, had absolutely no effect on the viscosity of either mucin or mucin–albumin mixtures (not shown). This finding did not make the calcium 'bridging' hypothesis attractive, and chelation experiments were therefore not pursued.

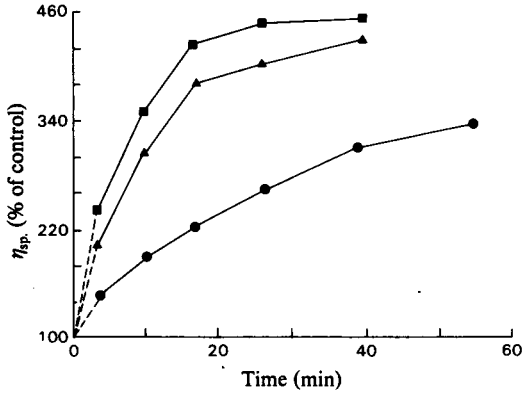

Fig. 6. *Effect of temperature on the viscosity of mucin and albumin*
Well-mixed samples of mucin (8mg/ml), albumin (20mg/ml) or mixtures of the two in 0.1M-KH_2PO_4/K_2HPO_4 buffer, pH7.4, were incubated with no shaking at 35 (●), 45 (▲) or 55°C (■) for 60min. At various time intervals viscosity was measured at shear rates of 11.25 and $22.5s^{-1}$. $\eta_{sp.}$ values of mucin–albumin mixtures at γ_w of $11.25s^{-1}$ are plotted as a percentage of mucin controls (arbitrarily set to 100%).

Both albumin (Lapanje, 1971) and mucins (Davis *et al.*, 1975) have been shown to contain hydrogen bonds sensitive to disruption by urea, and we therefore wished to see if urea might alter the viscosity of mucin–albumin complexes. Fig. 5 shows that 7M-urea caused a marked increase in the viscosity of mucin. At low shear rates albumin also underwent a slight (approximately 1 cP) increase in viscosity (not shown). Thus urea causes both molecules to unfold to produce a more extended three-dimensional configuration (Yang, 1961; Tanford, 1970). A large increase in viscosity was also observed in mucin–albumin mixtures with urea, and was proportionately greater at high than at low shear rates. Thus the addition of urea to mucin–albumin mixtures appears to facilitate mutual intertangling of fibres, but also tends to stabilize that interaction against the disruptive effects of high shear rate. The urea experiments did not provide evidence that hydrogen-bonding accounted for the initial viscosity-enhancing effect of albumin observed in Fig. 2. In fact, the major role of intramolecular hydrogen bonds may be the reverse, that is, to prevent excessive interaction of the two molecules.

Effect of heating

Both mucins (Allen *et al.*, 1974) and albumin (Spector, 1975) have been reported to be capable of

hydrophobic interactions. As pointed out by Kauzmann (1959) and Tanford (1970), many protein-aggregation reactions appear to depend on entropy-driven hydrophobic bonding reactions stabilized by heat. We therefore investigated the possibility that mucin–albumin interactions, as judged by viscosity, might also show a significant correlation with temperature. Incubations containing mucin, albumin or mixtures of the two, were carried out at different temperatures (35, 45 and 55°C) under very low shear conditions to maximize the interaction. At frequent time periods samples were removed and their viscosities measured at low shear rates (22.5 and 11.25 s^{-1}). In the case of both mucin and albumin alone, elevations of temperature produced a 10–15% decrease in viscosity, changes that were fully reversible on returning the temperature to 25°C (not shown). However, in mucin–albumin mixtures, temperature elevation had a dramatically different result (Fig. 6). There was an increase in viscosity with increased temperature, and the time required to reach the maximum elevation was achieved most quickly (25 min) at the highest temperature (55°C). For samples heated to 55°C, the viscosity changes could not be reversed by cooling to 20°C. These findings are compatible with the notion that mucin–albumin interactions are endothermic, stabilized by heat, and may depend in part on hydrophobic bonding. In view of the lack of reversibility of viscosity with cooling, however, it is also likely that heating caused some permanent configuration changes (denaturation) in one or both molecules, which favoured entanglement of the two.

Discussion

The experiments described in the present paper show that under conditions of physiological pH, temperature, iso-osmoticity, and at shear rates mimicking those of mucus secretions *in vivo* (Blake, 1975; Dulfano *et al.*, 1971), the addition of serum albumin to a model animal mucin produces a solution of high viscosity. The viscosity was higher than could be accounted for by a simple additive effect or by the calculated products of the viscosities measured separately. Similar findings were not obtained with α-casein, haemoglobin, collagen or human immunoglobulin G, suggesting that there was some specificity for albumin.

The molecular mechanisms by which mucin and albumin associate appear to involve non-covalent interactions that are readily, but reversibly, destroyed at high shear rates. Interaction was enhanced by heating, and was not significantly weakened by factors expected to disrupt ionic bonds, such as increases of pH, ionic strength or 7M-urea. Thus hydrophobic bonding may play a role in stabilizing mucin–albumin interaction. The hydrophobic areas of albumin that normally participate in the binding of fatty acids (Spector, 1975) may not be of prime importance in stabilizing mucin–albumin interactions, since fatty acid-containing or fatty acid-free preparations of albumin were indistinguishable in their action.

Because urea actually enhanced viscosity, and because the 55°C temperature-induced increase in viscosity was not reversed by cooling, it appears that partial denaturation and unfolding of mucin and/or albumin molecules enhances the interaction. Increased intertangling of unfolded fibres of mucin and albumin probably leads to a highly asymmetric, and thus viscous, complex.

Maximum enhancement of mucin viscosity occurred with an albumin/mucin ratio (w/w) of approx. 2:1. This is not a physiological ratio, since normal mucus secretions of the human respiratory (Potter *et al.*, 1963; Lamblin *et al.*, 1973), intestinal (Pecau & Feigelson, 1975) or reproductive (Masson, 1973) tracts contain little albumin. However, the present experiments may have relevance for pathological conditions characterized by high concentrations of albumin in mucus secretions (pulmonary infections, cystic fibrosis) (Potter *et al.*, 1963; Schachter & Dixon, 1965; Knauff & Adams, 1968). Serum exudation and stasis of mucus secretions (thus zero shear) would be expected to favour the association of mucin with albumin. In turn, the consequent elevation of viscosity may contribute to further obstruction.

A qualitatively similar phenomenon was observed by Fraser *et al.* (1972) for serum proteins and hyaluronic acid, the chief mucin component of joint synovial fluid. These authors have speculated that the gross elevation of mucin viscosity by serum protein may contribute to the increased work-load and stiffness of arthritic joints during periods of synovial effusions.

Whether human respiratory or intestinal mucins engage in the same interaction with albumin as the model pig mucin used in this study remains to be tested. Human intestinal mucin has been purified in our laboratory (Jabbal *et al.*, 1976), but is not yet available in sufficient quantity for multiple viscosity experiments. However, since the general chemical, physical and rheological properties of all glycoprotein mucins appear to be quite similar (Gibbons *et al.*, 1970; Gibbons, 1972; Pigman & Moschera, 1973), the pig mucin is felt to represent an adequate model for qualitative assessment of the behaviour of human mucins.

Financial support was obtained from the Canadian Cystic Fibrosis Foundation and the Medical Research Council of Canada. S. J. L. and B. P. F. were awarded summer student fellowships from the Canadian Cystic Fibrosis Foundation.

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