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SHEAR-INDUCED PROTEIN–PROTEIN INTERACTION AT THE AIR–WATER INTERFACE

J. G. WATTERSON, M. C. SCHAUB and P. G. WASER

Institute of Pharmacology, University of Zürich, Gloriastrasse 32, CH–8006 Zürich (Switzerland)

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SUMMARY

Increases in the viscosity of solutions of different proteins varying over a wide range of molecular shape and size have been studied in a cone and plate viscometer. The protein concentrations used were lower than those needed to produce viscosities of the solutions significantly greater than the solvent medium. The high values of shear stress finally reached are attributed to a shear-induced formation of an intermolecular structure resulting from protein–protein interactions at the air–water interface.

The rate of formation of this structure was found to increase with ionic strength, indicating that the rate of adsorption at the interface may influence the development of high shear stress values. However, this effect also shows that hydrophobic intermolecular interactions and the conformation of the protein molecules in solution are important factors in the development of the structure. The presence of small amounts of detergents was found to be able to prevent increases in shear stress. The fact that the double-chained lipid lecithin does not produce this effect, even in high concentrations, may be due to its ability to take part in protein–lipid–protein interactions.

INTRODUCTION

A strong influence of the air–water interface on viscosity measurements of protein solutions was recognized some decades ago [1, 2]. However, the nature of the interfacial phenomenon is still not well understood although the age and surface pressure as well as the particular method of measurement of the interface are frequently reported as being important factors.

On the other hand the technique of spread monolayers has yielded much information about protein surface films [3, 4]. The occurrence of various types of films [1, 2], including even a two-dimensional gel-like state [5, 6], or the formation of strong intermolecular bonds [7] have both been suggested to explain the wide variability in the properties of such monolayers. More recently, the results of rhe-

ological [8–10] and surface compression [11–14] studies have been viewed in terms of changes in the tertiary structure of the protein chains.

Considerably fewer investigations on protein films formed by adsorption at the interface from solution are to be found in the literature [15, 16]. The studies reported here were carried out on dilute protein solutions having a bulk viscosity not significantly different from that of the solvent, so that all observed effects may be attributed to the formation of a structure somewhere within the system. Work in progress in this laboratory shows that dilute solutions of not only protein- but also water-soluble acrylic and vinyl synthetic polymers show anomalous viscosity behaviour when measurements are made in the presence of an interface. This effect has been attributed to the adsorption of the solute macromolecules at the interface when the solutions are subjected to low shear [17].

MATERIALS AND METHODS

Proteins. Myosin was prepared from white skeletal muscles of rabbits using the method of Trayer and Perry [18] with the modification that 5 mM EDTA was used in all solvents apart from the extraction medium. Cleavage of the myosin molecule into its water-soluble fragment bearing the duplex globular head portion, heavy meromyosin, and its water-insoluble rod moiety, light meromyosin, was achieved by partial tryptic digestion. Separation of the two protein fragments was accomplished by selective precipitation [19]. To denature myosin samples of the protein were dissolved in 6 M guanidine · HCl, left for 4 h at 20 °C and subsequently dialysed exhaustively against 0.5 M KCl containing 25 mM Tris–HCl buffer, pH 7.6.

Tropomyosin was prepared from the same source by a modification [20] of the procedure of Bailey [21].

Collagen was extracted from pieces of rat tail tendons by slow stirring for 3 h at 2 °C and pH 3.8 [22]. The protein concentration was adjusted to about 1 mg/ml, the pH brought to 7.6 and any insoluble material filtered off. Collagen was denatured by warming samples to 60 °C for at least 30 min directly before the experimental runs.

Pepsin, three times recrystallized of B grade was purchased from Calbiochem, and bovine serum albumin, twice recrystallized from Fluka, Switzerland.

Reagents. All chemicals were of the highest grade obtainable. The disodium salt of ATP was purchased from Sigma. Distilled and deionized water was used throughout.

Viscosity measurements. Before use the freeze-dried proteins were dissolved in the appropriate media as indicated in the text. Samples of myosin and collagen were dialysed against the corresponding solvent before dilution for viscometry. Rate of shear values ranging up to 230 s^{-1} were produced in the Wells–Brookfield LVT model cone and plate viscometer with a cone angle of 1.565°. This instrument delivers a constant rate of shear throughout the bulk of the sample as well as across the air–water interface. The sample volume was 1 ml and the air–water interface is estimated to be about 1 cm^2, whereby the shearing force extends across a distance of 0.7 mm in this interface. Outflow runs were done using an Ubbelohde viscometer with an outflow time of 1.55 min for water at 25 °C.

Enzymic assay. The K^+-stimulated ATPase of myosin [23] was measured in

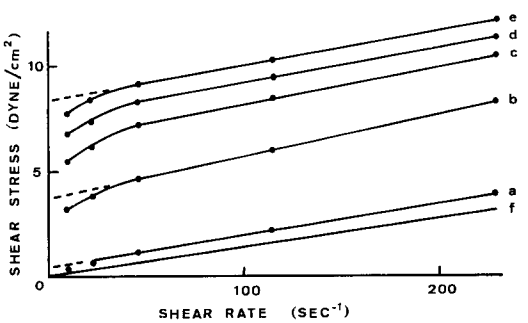
25 mM Tris–HCl buffer, pH 7.6, 10 mM EDTA and 1 M KCl as described elsewhere [24] at 8 °C, the temperature at which viscosity determinations were done.

Protein concentration. The concentration of proteins in solution was estimated by the biuret method standardized by nitrogen analysis with a micromethod involving nesslerization [25].

RESULTS

Protein concentration

A typical flow diagram illustrating the dependence on protein concentration is shown in Fig. 1. The readings are obtained at the various shear rate values indicated by the points after the instrument has begun to record a constant, maximum shear stress value. Diagrams of this nature, with curves having linear portions parallel to the straight line produced by the newtonian behaviour of the solvent alone, are by no means peculiar to myosin but were produced by each macromolecular solution so far investigated. A plot of the maximum viscosity reached against protein concentration exhibits a steep rise in shear stress up to a critical protein concentration, above which the curve levels off. In the case of myosin the maximum shear stress value, although some scatter is observed, remains constant between protein concentrations of 0.1–2.0 mg protein/ml. On the other hand the bulk viscosity of such protein solutions continuously increases over this concentration range. The contribution of the bulk viscosity to the overall shear stress registered at low shear rates was therefore calculated. The dashed line in Fig. 2 shows the value of the shear stress due to the solution at a shearing rate of 115 s^{-1}. The values were obtained by transforming the relative viscosities from outflow measurements to shear stress values corresponding to this constant velocity gradient. Thus over the entire concentration range shown the bulk viscosity does virtually not contribute to the high values of shear stress observed.



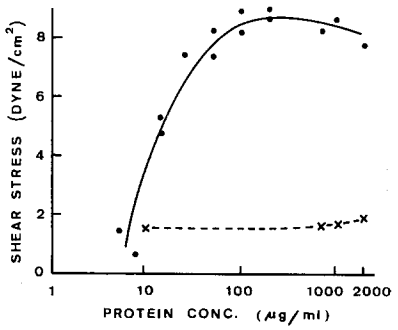


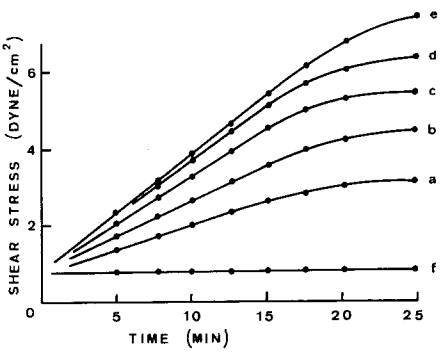
Fig. 1. Dependence of maximum shear stress on rate of shear for myosin solutions of different protein concentrations in 25 mM Tris–HCl, pH 7.6, and 0.5 M KCl at 8 °C. a, 9; b, 14; c, 50; d, 100; e, 200 µg myosin/ml; f, solvent alone.

Fig. 2. Dependence of maximum shear stress on protein concentration for myosin in 25 mM Tris–HCl, pH 7.6, 0.5 M KCl at shear rate 115 s^{-1} and 8 °C (full symbols). Crosses represent shear stress values calculated from outflow measurements done under identical conditions.

Effect of ionic strength

Since myosin is soluble only at relatively high ionic strength, i.e. above 0.3 M KCl, the effect of salt on the viscosity build up at low shear rates is illustrated for the case of the water-soluble protein tropomyosin (Fig. 3). The increase in the viscosity build up as well as higher final shear stress values with increasing KCl concentrations (up to 1 M) was generally found with all water-soluble proteins. This result is of particular interest since, in the case of tropomyosin the solution viscosity declines as the salt concentration is increased [26]. This is interpreted as the breakdown of the fibrous aggregates existing in the absence of salt into the double-stranded helical monomer species present at high ionic strength.

The effect of salt on solutions of native collagen is also shown, where increasing the ionic strength effects the rate of viscosity build up in a similar way (Fig. 4). However, when the KCl concentration is above about 50 mM the final values of the shear stress reached become similar. This effect seems to be the result of a sudden



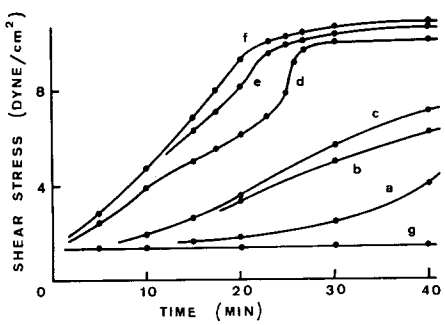


Fig. 3. Dependence of time-course of shear stress development on salt concentration for a tropomyosin solution of 40 μg protein/ml in water, pH 6.5, at 8 °C and shear rate 115 s^{-1}. a, 0; b, 0.05; c, 0.2; d, 0.5; e, 1.0 M KCl; f, water alone.

Fig. 4. Dependence of time-course of shear stress development on salt concentration for a native collagen solution of 0.1 mg protein/ml in water, pH 6.5, at 8 °C and shear rate 115 s^{-1}. a, 0; b, 10; c, 20; d, 50; e, 100; f, 500 mM KCl; g, water alone.

increase occurring just before the maximum level is reached in systems containing salt near this critical concentration. Nevertheless, the general feature observed with all water-soluble native proteins is that the higher the salt concentration the sooner the final level of shear stress occurs. The same behaviour was observed using samples of denatured collagen (Fig. 5). In this latter case, however, the rate of viscosity increase was not so markedly affected but rather the time lag before the sharp transition from low to high levels of shear stress, found in the absence of salt, was shortened. The experiments illustrated in Fig. 5 were carried out at 45 °C to prevent any renaturation [27]. The previous heating of the dilute collagen solutions at 60 °C is known to transform the triple-stranded helix into randomly coiled chains [28].

Since the cleavage of myosin by partial tryptic digestion enables the separation of a water-soluble protein fragment, heavy meromyosin, from the α-helical rod moiety, light meromyosin, [19] it can be examined whether the behaviour of the

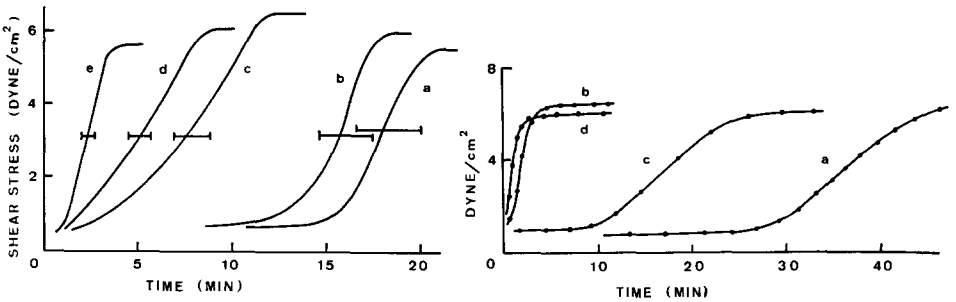


Fig. 5. Dependence of time-course of shear stress development on salt concentration for a denatured collagen solution of 0.1 mg protein/ml in water, pH 6.3, at 45 °C and shear rate 115 s^{-1}. Each curve represents an individual run, horizontal bar shows scatter in mid-point of transition in four runs. a, 0; b, 10; c, 50; d, 100; e, 500 mM KCl.

Fig. 6. Development of shear stress with time in solutions of myosin and its fragments in water, pH 6.8, at 8 °C and shear rate 115 s^{-1}. a and b, water-soluble heavy meromyosin, 0.02 mg/ml (a, 0 and b, 0.5 M KCl); c, water-insoluble rod fragment light meromyosin, 0.02 mg/ml in 0.5 M KCl; d, intact myosin, 0.05 mg/ml in 0.5 M KCl.

intact molecule is the result of the influence of the high-ionic strength medium mainly on one part of the molecule only. Fig. 6 indicates the rod moiety dissolved in high salt leads to a gradual viscosity build up with a rate comparable to those found with native tropomyosin and collagen under similar conditions. Increasing the salt concentration markedly affects the behaviour of the water-soluble portion of the molecule and the rate and onset of shear stress build up approaches that of the intact molecule at 0.5 M KCl.

Effect of temperature and pH

The dependence of shear stress value on temperature is shown in Fig. 7 for myosin dissolved in 0.5 M KCl. Readings were taken at each temperature after a

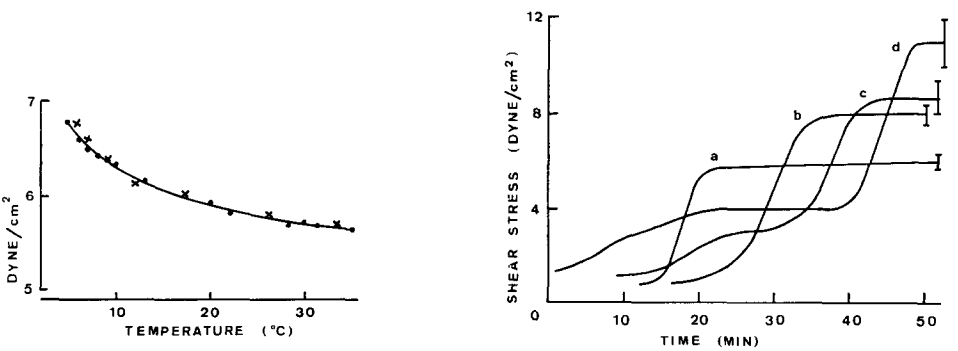


Fig. 7. Effect of temperature on maximum shear stress for myosin solution of 0.1 mg protein/ml in 20 mM phosphate buffer, pH 7.1, and 0.5 M KCl at shear rate 115 s^{-1}. Full circles, decreasing temperature; crosses, increasing temperature.

Fig. 8. Effect of temperature on time-course of shear stress increase of denatured collagen, 0.1 mg protein/ml in water, pH 6.5, and at shear rate 115 s^{-1}. Each curve represents an individual run, vertical bar indicates scatter of maximum value reached after 50 min in three runs. a, 45 °C; b, 35 °C; c, 25 °C and d, 8 °C.

steady-state value was registered. The temperature dependence is completely reversible and shows no indication of hysteresis. Such an experiment covering the temperature range from 5 to 35 °C in both directions extended over a time period of about 90 min. No significant temperature dependence of the time of onset of viscosity build up was found in the case of myosin. The most marked effect of temperature was found when samples of denatured collagen were quenched from 60 °C to lower temperatures ranging from 8 to 45 °C for viscosity measurements. The major sharp transition, occurring earlier the higher the temperature, can be seen in Fig. 8. The extent of this transition seems to be independent of temperature although the final level reached rises at lower temperatures.

Variation of the pH between 5.5 and 9.5 did not markedly affect the characteristics of the viscosity behaviour of the proteins studied.

Effect of low-molecular compounds

The presence of the surface-active compounds octyl-phenylpolyethyleneoxide (Triton X-100) and sodium dodecylsulfate prevent the fast shear-induced viscosity build up even at high ionic strength so that the protein solutions, up to concentrations of 2 mg/ml, behave like the solvent alone. This effect was found even at very low concentrations of the detergents. To determine the minimum concentration needed to prevent an increase in viscosity different ratios of detergent to protein were made up prior to the run. As Table I shows the number of Triton X-100 molecules required for the effect is of the same order as the number of protein molecules. In fact the molar ratio of detergent to protein is less than 1 in the cases of the smaller-molecular weight water-soluble proteins. For the high-molecular weight water-insoluble myosin a ratio higher than 1 was found. Although there seems to be a dependence of this ratio on the protein concentration, the concentration of detergent varies by a slightly smaller factor of about 20 as compared to one of 50 for the protein. The results with sodium dodecylsulfate gave similar mole to mole ratios. The double-chained lecithin molecule was found to have no effect whatever in similar concentrations and reduced the maximum shear stress value reached only fractionally when used in a molar concentration as high as 1000 times over that of protein. However, the additional presence of Triton X-100 or sodium dodecylsulfate in concentrations 10 times smaller than that of lecithin prevent any viscosity increase.

TABLE I

Concentration of Triton X-100 required to prevent shear stress increase of various protein solutions in 25 mM Tris–HCl, pH 7.6, 0.5 M KCl at 8 °C and shear rate 115 s^{-1} (averages of three experiments each). Molecular weight of Triton X-100 taken to be 640.

Protein	Molecular weight	Protein concentration (mg/ml)	Concentration of Triton X-100 (μM)	Moles of Triton X-100/ moles of protein
Pepsin	35 000 [29]	1.8	24	0.46
Bovine serum albumin	68 000 [30]	1.6	21	0.89
Tropomyosin	70 000 [31]	0.4	2.7	0.48
Myosin	470 000 [32]	2.0	9.4	2.2
Myosin	470 000	0.2	3.9	9.1
Myosin	470 000	0.1	2.1	10.0
Myosin	470 000	0.04	1.1	12.4

The polyanions EDTA, ATP and ethyleneglycol bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA) also affect the viscosity build up but only in sufficiently high concentrations in the millimolar range, i.e. about 1 000 000 times in excess of the molar concentration of protein. The results with EDTA are given in Table II where the relative values of the finally reached shear stress for the cases of myosin, denatured myosin and tropomyosin in 0.5 M KCl are shown. The effect of EDTA in preventing the development of high shear stresses is most pronounced with tropomyosin and least with native myosin. The reversal of this effect by the additional presence of similar amounts of divalent cations occurs only in the cases of native and denatured myosin. Since similar effects were found using other proteins, i.e. albumin and pepsin, they seem to reflect a general feature of such polyanions.

TABLE II

Effect of EDTA and Mg^{2+} on maximum shear stress of myosin and tropomyosin solutions in 25 mM Tris–HCl, pH 7.6, 0.5 M KCl at 8 °C and shear stress 115 s^{-1} (averages of three experiments each).

Protein	EDTA (mM)	$MgCl_2$ (mM)	Relative shear stress (%)
Native myosin	0	0	100
(0.1 mg/ml)	7.5	0	9
	10	2	23
	2.5	0	37
	2.5	2.5	72
	0	2	98
Denatured myosin	0	0	100
(0.1 mg/ml)	2.5	0	0
	0.5	0	9
	1	3	83
	0	3	95
Tropomyosin	0	0	100
(0.04 mg/ml)	2	0	0
	2	2	0
	2	5	0
	0	2	103

The effect of these low-molecular weight compounds on the bulk viscosity of proteins was investigated by outflow viscometry at high ionic strength. These experiments were performed on protein solutions ranging from 0.5 to 5 mg of protein/ml. The results obtained indicate that all low-molecular weight compounds used so far have no measurable effect on the bulk viscosity of the protein solutions.

DISCUSSION

Since the concentrations of protein used in these studies are too low to produce any significant differences in the bulk viscosity of the solvent, in all cases an aqueous salt solution, the observed increases detected in the cone and plate viscometer must be attributed to a structure build up somewhere in the system. That this phenomenon

probably occurs at the air–water interface, which in this instrument extends from the rotating cone to the stationary plate and is therefore itself under shear, is supported by the experiments reported by Blank [9], where the protein was introduced onto the air–water interface by a spreading technique.

Although diffusion of solute molecules to the interface must take place, the fact that the time effect found in all systems whereby the shear stress always increases with time, is unlikely to be entirely due to a diffusion-controlled rate of adsorption at the interface, is supported by the following arguments. (1) Parallel findings have been reported [9] for systems in which the protein is actually spread on the interface so that the question of adsorption does not arise. (2) In many cases a definite period of shearing is required after which the effect suddenly takes place.(3) It is improbable that low concentrations of detergent prevent the adsorption of macromolecules while considerably higher concentrations of lecithin do not. (4) Protein adsorption from aqueous solutions in the absence of salt is complete within a few minutes [16]. (5) Even in the presence of salt and even though the system is obviously constantly stirred, as long as 20 min may be required to reach the final shear stress values (see Figs 3 and 4).

If there is no structure formation in the bulk of the solution it must have the same viscosity as the solvent. This fact is demonstrated in Fig. 1 where the slopes of the curves equal that of the curve of the solvent alone over a wide range of the velocity gradient. In other words, the viscosity of each solution, if understood as being the increase in shear stress offered by the system for unit increase in shear rate, is just that of the solvent medium. The contribution of the surface to the overall final viscosity is therefore given by the intercept on the shear stress axis. Since the value of the intercept increases with increasing protein concentration in the bulk phase up to a saturating level at about 0.1 mg/ml, it may be taken as a measure of the amount of material adsorbed in this concentration range.

In general the values of the surface viscosity of protein systems is considered to be abnormally high [34]. They are often some 100- or 1000-fold higher than solution viscosities even when measured at surface pressures where it is claimed the surface viscosity is newtonian [15]. This is illustrated in Fig. 1 by the curvature exhibited by the flow curves below rate of shear values of about $20\ s^{-1}$. In this lower range the protein molecules may be thought to form a network of high viscosity. On the other hand, higher-velocity gradients are able to break this interconnected structure and the interfacial phase now flows with a shear viscosity of less than 0.01 surface poise. However, the interaction between the adsorbed molecules remains sufficiently strong so that the high shear stress values are maintained at the higher rates of shear.

The lowering of the ζ-potential of dissolved macromolecules by addition of salt diminishes the long-range electrostatic interactions so that the bulk phase viscosity is usually decreased while the adsorption rate is markedly increased [7, 15, 16]. Thus one would expect a closer molecular packing and higher rate of intermolecular collison at the interface [16]. These considerations are born out by our results in that the addition of KCl invariably resulted in a faster build up of the shear stress and sometimes in a higher shear stress value finally reached. However, as mentioned above, the simplified idea of a diffusion-controlled adsorption rate does not explain the results because the time needed for shear stress build up is too large, being often greater than 10 min. Thus the increase in ratio of the strength of hydrophobic to

hydrophilic interactions resulting from the increase in salt concentration may be the most important factor as argued elsewhere [17].

The behaviour of tropomyosin is particularly illustrative in this respect since the bulk viscosity of this protein falls upon addition of salt, an effect interpreted as the breakdown of larger assembled protein structures to smaller following the disruption of intermolecular salt bridges [26]. On the other hand the type of viscosity behaviour reported here is promoted by salt, implying that intermolecular interactions of a kind other that electrostatic can be brought into play at an interface when subjected to shearing forces. These results make it unlikely that the association of monomeric species into extensive aggregates is responsible for the high shear stress values as has been found in systems of high-molecular weight polymers in the extremely viscous solvent "Aroclor" [35–37]. Furthermore, no indication of any gel formation nor any drop in high shear stress readings when shearing was stopped for periods of up to 30 min, was ever observed.

The difference between the response of tropomyosin and native collagen to increasing ionic strength on the one hand and of pepsin, heavy meromyosin and denatured collagen on the other, probably reflects the difference in shape and tertiary structure between the two general classes of proteins. The former are rod-shaped molecules and are not as dramatically affected by salt as the latter group of globular and random-coiled molecules. In a salt medium the overall charge of water-soluble macromolecules falls [38]. In the case of globular or random coil conformation this results in a contraction of the molecule. On the other hand rods would not be expected to change shape unless the salt effect is drastic enough to rupture the interchain interactions which give the double- and triple-stranded rods, in the cases of tropomyosin and collagen, respectively, their stability. The fact that MacRitchie and Alexander [16] found no marked influences of molecular shape or size on the kinetics of adsorption is possibly due to the relatively high ionic strength in the substrate phase used. The marked influence of salt on globular in contrast to rod conformation is also illustrated by the behaviour of intact myosin which exhibits a rate of shear stress build up similar to that of its globular moiety heavy meromyosin alone.

Surprisingly small concentrations of detergents are required to abolish the shear stress build up. One possible explanation is that the presence of these compounds lowers the chemical potential of the protein molecules in the aqueous medium sufficiently so that they have no longer the tendency for preferential adsorption at the interface. However, it is more likely that, after adsorption of the detergent molecules onto hydrophobic regions of the protein, the hydrophilic moiety of the detergent molecules so introduced prevent the protein–protein interaction. That other small molecular weight compounds such as EDTA are also able to reduce the viscosity build up may also be due to their association with the protein molecules because of their partial hydrophobic character. Nevertheless other structural features of the detergent also appear to play a role, since the double-chained lecithin molecule was found to have no effect whatever in similar concentrations to those of Triton X-100 and sodium dodecylsulfate, and reduced the maximum shear stress value reached only fractionally when used in concentrations as high as 100 μM irrespective of the protein in the system. This result is of particular interest since lipid monolayers are known to enhance the rate of protein adsorption [39] which is thought to involve the penetration of the protein into the surface. Thus although protein–lipid interactions are

very likely present, no plasticizing effect due to the lipid was observed in this work. However, the added presence of 10 μM Triton X-100 was again found able to prevent any structural formation from occurring. This ability of detergents to destroy the protein–protein and protein–lipid interactions, although only perhaps with the help of shearing forces, may be correlated with the usefulness of these agents in the extraction and preparation of the constituents of biological membranes. Although lipids such as lecithin exhibit the general physical properties of detergents, their double-chain nature seems to allow them to associate with two individual hydrophobic regions leading to an interconnected network. Thus if the lecithin molecules do intervene in the regions of protein–protein interaction one may envisage that it is replaced by a protein–lipid–protein type of interaction. This would imply that lipid molecules of more than one aliphatic chain are necessary for membranous structures to sustain shearing forces.

Although no deterioration of the enzymic activity of myosin was detected after subjection to shear the possibility of denaturation in some cases is recognized. Effects found in surface-tension investigations are often ascribed to chain unfolding and even protein coagulation [13, 15]. On the other hand strong evidence has been presented to show that peptides in the α-helical conformation are able to build extensive structures at an interface without denaturation [40, 41]. Comparison of the results presented here with those from systems of soluble synthetic copolymers [17] which adopt some form of random-coil conformation in solution, shows that the randomly coiled denatured collagen systems behave in a similar way to those of the synthetic polymers. The main feature of similarity is the time lag before the onset of increase in shear stress which is shortened in the presence of salt. The different behaviour displayed by the systems in which the proteins were originally in the native state may indicate that no extensive denaturation occurred during examination. However, even if the molecules remain in the native state the effect of shear would be expected to result in a mutual orientation which brings strong interactions into play. And although these interactions exhibit a high resistance to shear this cannot be the result of the intertwining of unravelled chains because of the alignment produced by the shearing force.

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