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Gelation of Plasma Proteins

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ABSTRACT

The rheological properties of plasma protein gels were markedly affected by protein concentration, pH, heating temperature and time and measuring temperature.

Gel strength increased with increasing protein concentration (6–18%), pH (4·0–9·0), heating temperature (60–90°C) and time (15–60 min), but decreased with increasing measuring temperature (10–60°C).

The ability of gels to recover following cyclic loading and unloading was low in the pH region 4·0–6·0, but increased at higher pH. Recovery increased with increasing measuring temperature, but decreased at heating temperatures above 75°C and with increased duration of heating (15–60 min).

INTRODUCTION

Studies relating to the rheological properties of plasma gels have concentrated on the effects of pH, heating temperature and ionic strength of the aqueous environment on the physical properties of such gels. Hermansson (1978) found that heating to 75°C was necessary for formation of strong gels from 4% w/v plasma protein solutions, although a thickening was noticed at 70°C. Similar temperature dependencies were reported by Harper *et al.* (1978) who found that on heating 12% w/v plasma protein

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solutions at 50 or 60°C no appreciable gelation occurred and strong gels did not develop until temperatures of 70–75°C were reached.

The marked influence of pH on the rheological properties of plasma gels has also been noted. However, different workers have reported maximum gel strength at different pH values (Fretheim & Gumpen, 1978; Hickson *et al.*, 1980; Hermansson, 1982*a*). The aim of this study was to investigate the effects of pH, heating temperature and time, heating rate, protein concentration, measuring temperature, ageing temperature and time on the viscoelastic and textural properties of plasma gels and to determine optimum conditions for plasma gel formation.

MATERIALS AND METHODS

Preparation of plasma

Lyophilised, dialysed plasma protein was prepared as described by O'Riordan *et al.* (1988).

Protein solubilisation

The dried plasma was solubilised in 0·1M imidazole. Protein content was determined by the Biuret method and pH was adjusted to the desired values using 1M HCl or NaOH.

Preparation of plasma gels

Aliquots (1 ml) of the plasma protein solution were transferred to glass tubes (inside diameter 6·0 mm), which had been treated with Sigma cote (Sigma Chemical Company, St. Louis, MO) and air-dried. The tubes containing the gelling solutions were centrifuged at low speed to remove dissolved gas and then heat-sealed to prevent evaporation. The samples were generally equilibrated in a water bath at 40°C for 15 min and the temperature of the bath was then raised to the required temperature in the range 60–95°C at a constant rate of 3°C min^{-1} and maintained at the required temperature for a fixed period of time which varied from 15 to 60 min. In some instances, the tubes containing gelling solutions were heated immediately to the required gelling temperature by immersion in a water bath at that temperature. The time for the protein solution to reach the required gelling temperature was determined by means of a thermocouple placed in the geometric centre of the tube and was found to be <1 min. Following heating, the tubes were cooled by immersing them in water at different ageing temperatures in the

range 4–42°C and were then held at the desired temperature for up to 30 h prior to removal of the gels and assessment of their rheological properties.

Measurement of gel rheological properties

The gels formed in the glass tubes were carefully removed and sections 5 mm high were cut with a razor edge cutting device. The rheological properties were determined with a Rheoner RE-3305 (Yamaden Co., Ltd., Tokyo) fitted with a 2 kg load cell to which a circular pressure plate (16 mm in diameter) was attached. To conduct measurements at room temperature ($\sim$25°C) a gel section (5 mm $\times$ 6 mm diameter) was placed on the base plate of the Rheoner and the plate was moved in an upward direction towards the pressure plate at a speed of 0·5 mm s^{-1}. The gel sample was compressed between the two plates on two consecutive occasions by either 20% (1 mm) or 70% (3·5 mm) of its original height. The force exerted on the pressure plate was sensed by the load cell and recorded continuously.

In order to determine the effects of measuring temperature (10–60°C) on the rheological properties of plasma gels, the normal base plate was replaced by a double walled temperature-controlled measuring platform containing paraffin. The temperature of the sample (10–60°C) was maintained by circulating water from a constant temperature bath through the jacket of the platform. The samples were allowed to equilibrate at the desired temperature for 1 h prior to measurement.

The following rheological parameters were determined from the force–deformation curves as described by Mulvihill & Kinsella (1988):

Stress at 20% compression ($\sigma_{0\cdot2}$), units = N/m^2
Apparent modulus of deformability (AMD), units = N/m^2
Degree of elasticity ($DE_{0\cdot2}$), units = ratio, dimensionless
Mechanical hysteresis loss (MHL), units = %
Stress at 70% compression ($\sigma_{0\cdot7}$), units = N/m^2
Elasticity/springiness ($E_{0\cdot7}$), units = ratio, dimensionless.

All rheological results reported are mean values calculated from a minimum of six and up to twelve separate force–deformation curves obtained from different gel sections.

RESULTS AND DISCUSSION

The effects of protein concentration (6–18% w/v) on the rheological properties of plasma gels were studied. At pH 9·0 the minimum protein concentration required for the formation of self-supporting plasma gels was

6% w/v protein. The parameters relating to the strength of the plasma gels ($\sigma_{0.2}$, $\sigma_{0.7}$, AMD), shown in Fig. 1(a), increased with increasing protein concentration in an almost linear manner. These findings are consistent with the work of Hermansson (1982*b*). The observed increase in gel strength is attributed to the increased density of the protein phase, resulting in more sites of interaction and increased crosslinks.

The ability of plasma gels to dissipate stress energy was independent of protein concentration, as evidenced by the MHL values (Fig. 1(b)). However, $DE_{0.2}$ values (Fig. 1(b)), reflecting the ability of the gel to recover following deformation, increased substantially with increasing protein concentration

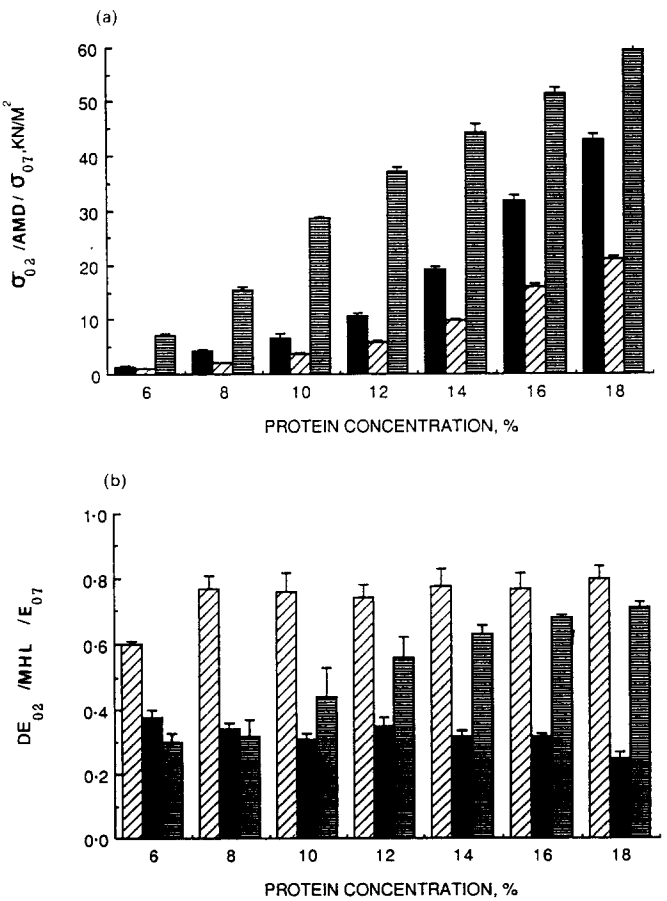

Fig. 1. Effects of protein concentration on the rheological properties of plasma protein gels: (a) stress at 20% compression (■), $\sigma_{0.2}$ (kN/m^2); stress at 70% compression (▤), $\sigma_{0.7}$ (kN/m^2 × 10^{-1}); apparent modulus of deformation (▨), AMD (kN/m^2 × 10^{-1}); (b) degree of elasticity at 20% compression (▨), ($DE_{0.2}$); mechanical hysteresis loss (■), $MHL_{0.2}$ (% × 10^{-2}) and springiness/elasticity at 70% compression (▤), $E_{0.7}$.

from 6 to 8%, but then remained relatively constant, or increased slightly at higher protein concentrations. $E_{0\cdot7}$ values (Fig. 1(b)) increased in an almost linear fashion with increasing protein concentration. However, this latter trend was possibily a consequence of fracturing of weak gels when subjected to 70% compression while the former parameters, $DE_{0\cdot2}$ and MHL, were measured by deformation below the fracture point of the gels and possibly better reflect the elastic nature of the gels.

The parameters relating to gel strength calculated from force–deformation curves obtained following both 20 and 70% compression of the gel samples ($\sigma_{0\cdot2}$, $\sigma_{0\cdot7}$, AMD) (Fig. 2(a)) increased with increasing pH in the range 4·0–9·0.

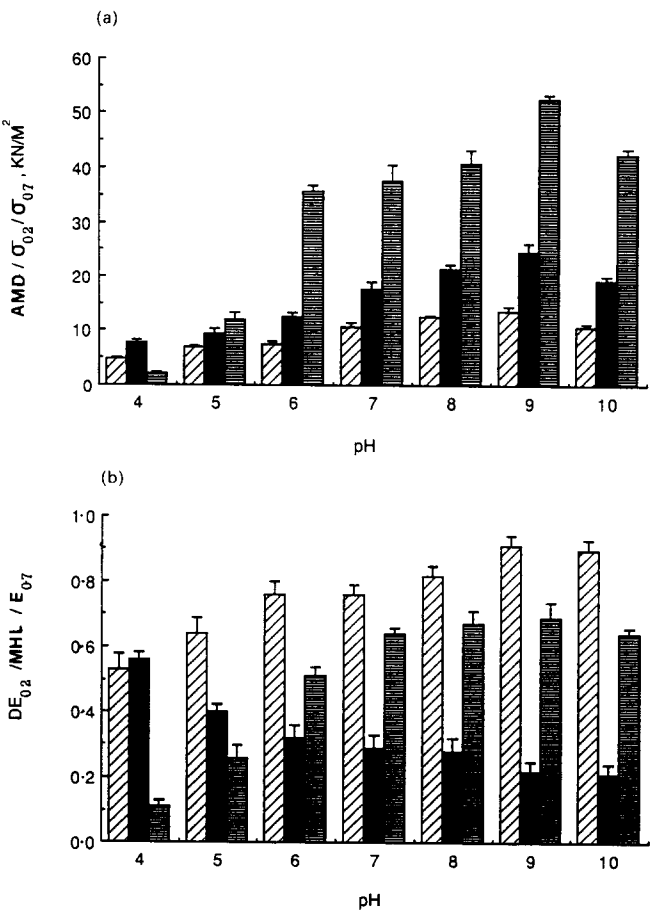

Fig. 2. Effects of pH on the rheological properties of plasma protein gels: (a), apparent modulus of deformation (▨), AMD (kN/m^2 × 10^{-1}); stress at 20% compression (■), $\sigma_{0\,2}$ (kN/m^2); stress at 70% compression (▤), $\sigma_{0\,7}$ (kN/m^2 × 10^{-1}); (b) degree of elasticity at 20% compression (▨), $DE_{0\,2}$; mechanical hysteresis loss (■), $MHL_{0\cdot2}$ (% × 10^{-2}) and springiness/elasticity at 70% compression (▤), $E_{0\,7}$.

This suggests that in the isoelectric region of plasma protein (pI = 5·0) the low net charge resulted in excess protein–protein interaction, and rapid protein association on heating, leading to large protein aggregates with weak interactive forces interspersed with regions of water. Increasing the pH above the isoelectric point resulted in firmer gels. This indicates that as the net negative charge increased, repulsion increased simultaneously, facilitating protein unfolding and alignment of protein strands, resulting in a firmer, more structured, gel with greater compressive strength.

Maximum gel strength was observed at pH 9·0, which coincides with the pH at which the albumin molecule expands, unmasking 150 labile hydrogen atoms (Peters, 1985). It is possible that these hydrogen atoms engage in hydrogen bonding, stabilising the protein matrix and increasing the gel strength. Hermansson (1982*a*) also observed optimum plasma gel strength at pH 9·0. However, Fretheim & Gumpen (1978) and Hickson *et al.* (1980) reported pH optima of 6·2 and 7·0, respectively. These discrepancies are possibly due to variations in the methods used to assess gel hardness. In the present study a decrease in gel strength was observed at pH 10·0, suggesting that, above the optimum pH, excessive repulsive forces reduce protein–protein interactions, resulting in a weaker gel.

The plasma gels formed at pHs 4, 5 and 6 exhibited poor ability to recover following deformation, as evidenced by $DE_{0.2}$ and $E_{0.7}$ values (Fig. 2(b)). This is characteristic of protein coagula (Ferry, 1948). The ability of the gels to recover increased at higher pH, possibly due to the formation of a more ordered structure combined with the fluidity of the entrapped water, in contrast to the syneresing structures formed in the pH range 4–5. The energy dissipated by the gel in cyclic loading and unloading decreased with increasing pH in the range studied, as evidenced by decreasing MHL values (Fig. 2(b)). In contrast to the effect of pH on gel strength, it is apparent that increasing the pH from 8·0–10·0 had little effect on the parameters relating to gel recovery following compression. This suggests that the increase in hardness values at alkaline pH may have resulted from the formation of covalent bonds, which when broken do not reform, and therefore would not contribute to the ability of the gel to recover.

In this study a minimum heating temperature of 60°C was necessary for the formation of plasma gels and at this temperature a heating period of 45 min was required for the formation of self-supporting gels. The rheological properties of gels formed at 60°C are not presented, since it was difficult to obtain accurate data due to the weak nature of these gels. Differential scanning calorimetry measurements showed that the onset of the denaturation peak for pure serum albumin began at 55°C, although the peak maximum occurred at higher temperatures (Leibman *et al.*, 1975; Gumpen *et al.*, 1979). Microcalorimetric studies of fibrinogen showed two

temperature transitions; one peak at 55°C and a second at 85°C (Medved *et al.*, 1980). Apart from serum albumin and fibrinogen, blood plasma contains several other proteins which may contribute to the gel structure. However, it is apparent that denaturation of the plasma protein precedes gel formation, suggesting a two-step process, as proposed by Ferry (1948).

A marked increase in values relating to gel compressive strength ($\sigma_{0\cdot2}$, $\sigma_{0\cdot7}$ AMD) (Fig. 3(a), (b), (c)) was observed on increasing heating temperature from 60 to 65°C. This suggests that, above the unfolding temperature, entropically-induced disruption of the native state occurs suddenly with likely exposure of at least some hydrophobic parts of the molecule to the solvent. Thus, regions of the molecule originally involved in stabilising the native form become available for intermolecular associations, resulting in the formation of a three-dimensional network. These results are consistent with the work of Harper *et al.* (1978), who reported the onset of plasma gelation at 65°C, while Hermansson & Lucisano (1982) reported that it was not possible to measure gel characteristics of 5% plasma protein solutions heated below 72°C.

The rheological properties relating to gel strength continued to increase with increasing temperature in the range 70–90°C and with increasing heating time (15–60 min) (Fig. 3(a)–(c)). Plasma proteins contain numerous disulphide bonds; BSA alone has seventeen intramolecular disulphide bonds. It is likely that a combination of alkaline pH and high temperature treatment results in cleavage of some disulphide bonds (Andersson & Berg, 1969: Shimada & Matsushita, 1981), leading to increased unfolding of the partially denatured plasma protein molecules. As a result the newly generated SH groups would be available for mercaptan interchange and further exposure of hydrophobic regions would lead to more intermolecular hydrophobic interactions, and hence stronger gels. The involvement of covalent disulphide bonds in stabilising the three dimensional network of plasma protein gels has previously been proposed by Howell & Lawrie (1985), and numerous workers have invoked the importance of disulphide bonds in BSA gels (Tombs, 1970; Richardson & Ross-Murphy, 1981; Shimada & Matsushita, 1981; Yasuda *et al.*, 1986).

The ability of the plasma protein gels to recover following deformation, as evidenced by $E_{0\cdot7}$ values (Fig. 3(d)), decreased slightly with increasing heating temperature above 75°C and with the increased duration of heating (15–60 min). This possibly reflects the presence of large diffuse aggregates of plasma protein following heating at 92°C compared to a regular network on heating at 77°C (Hermansson, 1982*b*). The increase in plasma gel elasticity with increasing heating time at 65°C (Fig. 3(d)) is possibly due to an increase in non-covalent bonds.

It is generally accepted that when heating conditions are extreme,

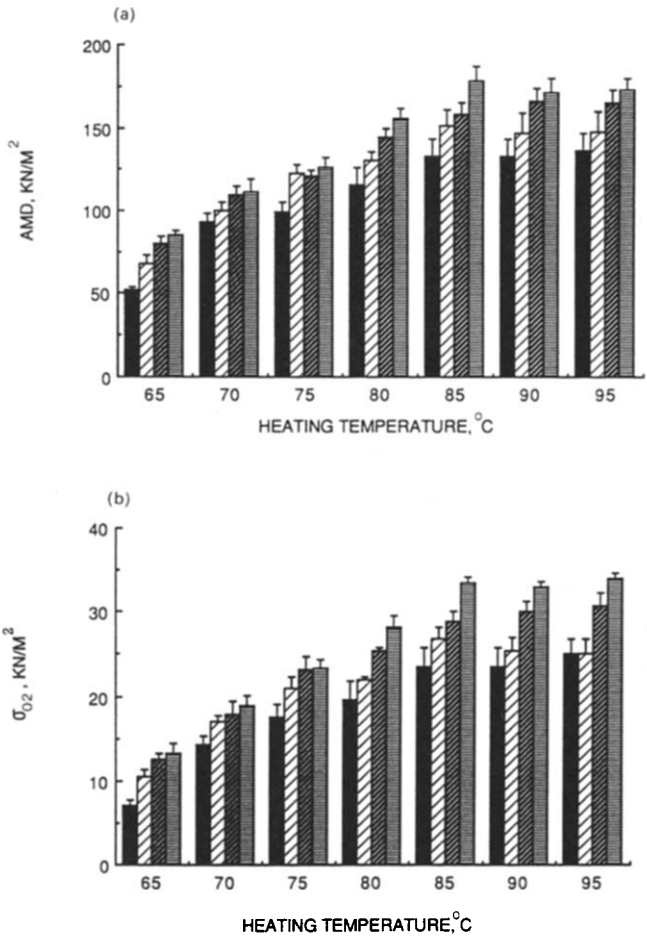

Fig. 3. Effects of heating temperature on (a) apparent modulus of deformation, AMD (kN/m^2); (b) stress at 20% compression, $\sigma_{0\,2}$ (kN/m^2).

denatured protein molecules may not have time to align themselves in an ordered fashion, and under these circumstances poorly hydrated aggregates or precipitates, which lack the continuous matrix of gels, are formed. Hence, it is believed that heating rate has a profound effect on gel characteristics (Kinsella, 1984). The results of this study indicate, however, that heating rate had no major effects on the rheological properties of plasma gels formed in the temperature range 75–95°C (data not shown). Foegeding *et al.* (1986) also concluded that heating rate in the temperature range 50–95°C did not affect the formation of BSA gels, although the heating rate did influence fibrinogen gels.

The rheological properties of gels formed by heating at 90°C and which were measured over a range of temperatures (10–60°C) are presented in

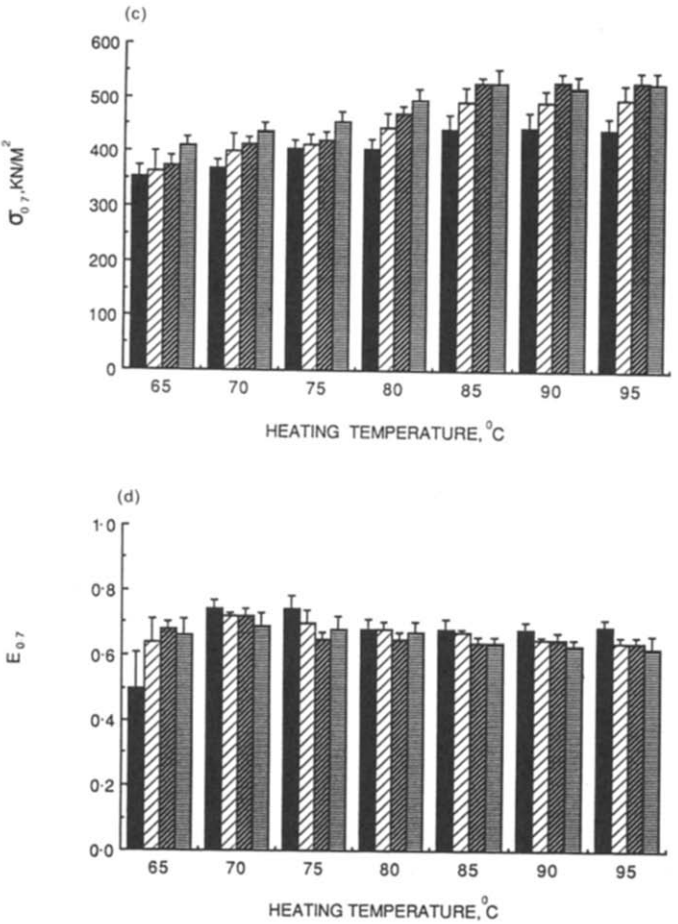

Fig. 3.—*contd.* (c) stress at 70% compression, $\sigma_{0\cdot7}$ (kN/m^2); and (d) springiness/elasticity at 70% compression, $E_{0\cdot7}$; of plasma solutions heated for 15 (■), 30 (▨), 45 (▨), or 60 (▤) min.

Fig. 4. Gel strength decreased with increasing measuring temperature, as evidenced by AMD, $\sigma_{0\cdot2}$ and $\sigma_{0\cdot7}$ values (Fig. 4(a)). However, the ability of the plasma gels to recover following deformation increased marginally with increasing measuring temperature, as indicated by MHL, $DE_{0\cdot2}$ and $E_{0\cdot7}$ (Fig. 4(b)). This suggests that plasma protein gels are stabilised by weak thermolabile bonds which rupture easily, but exhibit good ability to recover following deformation.

Varying the ageing temperature (4–42°C) and duration (1–30 h) of ageing had no major effects on the rheological properties of plasma gels (data not shown). Hence, plasma gel formation and stabilisation appear to occur during heating of the plasma solutions.

Overall, the results of this study suggest that heat-induced gelation of

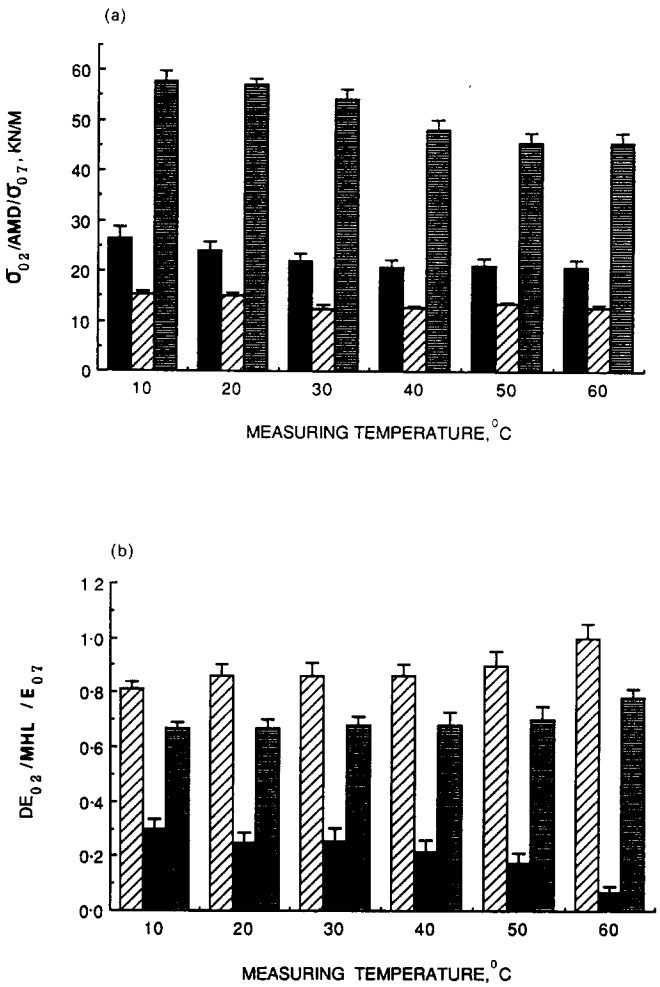

Fig. 4. Effects of measuring temperature on the rheological properties of plasma gels: (a) stress at 20% compression (■), $\sigma_{0\,2}$ (kN/m^2); stress at 70% compression (▤), $\sigma_{0\,7}$ (kN/m^2 × 10^{-1}); apparent modulus of deformation (▨), AMD (kN/m^2 × 10^{-1}); (b) degree of elasticity at 20% compression (▨), $DE_{0\cdot2}$; mechanical hysteresis loss (■), MHL (% × 10^{-2}), and springiness/elasticity at 70% compression (▤), $E_{0\,7}$.

plasma protein involves denaturation and unfolding of the native protein and limited protein association. This protein association is modified by the charged state of the molecule and is possibly directed by hydrophobic interactions. It is likely that this is followed by sulphydryl–disulphide interchange and intermolecular hydrogen bonding which also contribute to the gel matrix. Further studies are ongoing to confirm the molecular forces involved in the formation and stabilisation of plasma protein gels.

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