# Heat-Induced Gelation of Globular Proteins. 2. Effect of Environmental Factors on Single-Component and Mixed-Protein Gels

**Atsumi Tobitani[†] and Simon B. Ross-Murphy***

*Biopolymers Group, Division of Life Sciences, King's College London, Campden Hill Road, London W8 7AH, U.K.*

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ABSTRACT: In the first paper in this series, we investigated heat-induced gelation of single component systems of the globular protein, bovine serum albumin. A series of rheological experiments revealed that we can describe gelation time in terms of two physical parameters, concentration and temperature. In this paper we examine more complex systems, including the effect of pH and ionic strength, on heat-induced gelation of bovine serum albumin (BSA), and another globular protein, $\beta$-lactoglobulin ($\beta$-Lg). These factors significantly influenced protein gelation, a result which has not been reported in detail before. However, no detailed analysis was possible, because the behavior is very complex. We also consider the extension of both model and experiments to (quasi-) binary mixed systems, *viz*. the gelation of binary systems chosen from three globular proteins: BSA, $\beta$-Lg, and α-lactalbumin (α-La). We have obtained reliable results only for the BSA and $\beta$-Lg system because of experimental difficulties. From the data of the BSA/$\beta$-Lg system, it was found that gelation behavior changed significantly with the ratio of the two proteins. The change in the gelation time and temperatures was not expressed as a linear equation but required a cross-term. This strongly suggests that current models for the concentration dependence of modulus for mixed biopolymer gels will need to be modified.

## 1. Introduction

In the first paper in this series,[1] we have investigated heat-induced gelation of single component systems of the globular protein bovine serum albumin. A series of rheological experiments revealed that gelation time can be described in terms of two physical parameters, concentration and temperature. It was found from the analysis that gelation kinetics was explained satisfactorily by our model, and the "state" of the proteins, whether sol or gel, was presented as a function of these parameters in a diagram.

The purpose of this paper is to examine more complex systems. First, we consider the effects of two environmental factors, i.e. pH and ionic strength, on heat-induced gelation of bovine serum albumin (BSA). These have been known to affect the gelation process significantly and often cause problems in industrial use of protein gels, for example in biotechnology and food processing. Although a certain number of studies have been reported so far, the detail of the effect of such factors on the gelation mechanism is by no means fully understood. Second, we will consider the extension to (quasi-) binary mixed systems. In particular we will investigate the gelation of binary systems chosen from three globular proteins: BSA, $\beta$-lactoglobulin ($\beta$-Lg), and α-lactalbumin (α-La).

One of the early models for predicting the modulus of phase-separated biopolymer mixed gels is that due to Clark and co-workers.[2] This was constructed by considering the effect of solvent (water) redistribution between each gelling component and relating polymer concentration to the gel modulus, first to the component systems, and then by applying blending laws to the resultant composite gel. They used this model to analyze the rheological data for the agar/gelatin system, as well as microscopic observations, and demonstrated that it applied reasonably well. This model has since been used and tested for many mixed biopolymer systems,[3] for example by Walkenstrom and Hermansson,[4] and also modified by taking account of possible gel deswelling contributions.[5]

Although such a model can predict the relationship between the protein concentration and the gel modulus, it does not give any information about gelation points for mixed systems. Indeed, there seem no models which can be applied to describe the incipient gelation behavior of mixed systems. A number of previous studies have been published addressing all of the above problems. However, since many of the data contained complicated effects of several factors involved in sample preparation and measurement, it is very difficult to draw a clear conclusion from them. Consequently, few studies discuss the effect of the various factors in much detail, and none have considered the kinetic aspects, in particular the gelation time, as a major critical parameter for measurement. To investigate the effects of environmental factors on protein gelation, we have to design more systematic experiments.

The model developed in Part 1[1] of this series allows us, for the first time, to develop a more detailed understanding of such different and, undeniably, complex systems. It has also confirmed that gelation time measurements by isothermal heating are both sensitive and accurate. The gelation time thus determined can be used as an appropriate representative parameter of the gelation process and also is relatively easy to analyze in terms of reaction kinetics. For these reasons, we have examined the effects of pH and salt by means of gelation time measurement by isothermal heating.

## 2. Experimental Section

**2.1. Materials.** Bovine serum albumin (BSA), was the same material as in Part 1 (grade A-4378), but only Lot No. 13H9338 was chosen. The other globular proteins used were $\beta$-lactoglobulin ($\beta$-Lg) from bovine milk, 3 × crystallized and

* Corresponding author: Telephone: +44 (0)171-333-4081. Fax: +44 (0)171-333-4082. E-mail: simon.ross-murphy@kcl.ac.uk.
[†] Permanent address: Snow Brand Milk Products Co. Ltd. Technical Research Institute, 1-1-2, Minamidai, Kawagoe, Saitama 350, Japan.
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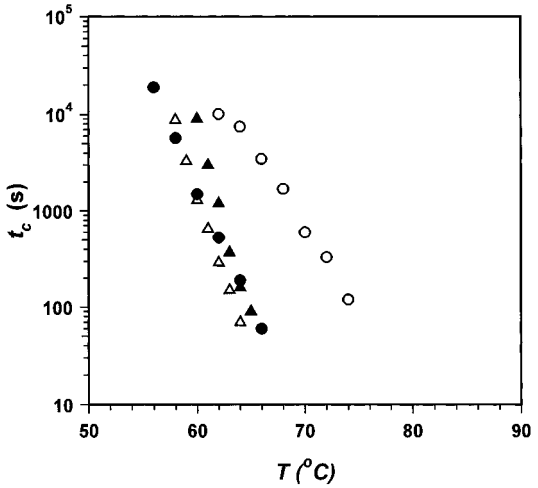


**Figure 1.** Gelation time vs temperature for BSA 8% w/w. Symbols correspond to different values of the salt ratio parameter $R$: open circles, $R = 9$ (native protein sample); filled circle, $R = 20$; open triangle, $R = 50$; filled triangle, $R = 100$.

lyophilized, L-0130, Lot No. 51H7210, and α-lactalbumin (α-La) type III, from bovine milk, approximately 85% polyacrylamide gel electrophoresis, 6010, Lot No. 128F8140. All were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Sodium chloride (S-9888) and sodium hydroxide (S-0899) were of analytical reagent grade from Sigma Chemical Co. Hydrochloric acid was from FAS Laboratory Supplies (Product No. 1789, Loughborough, U.K.). These materials were used without further purification.

**2.2. Methods.** The effect of salts was examined with sodium chloride. According to the manufacturers data, BSA used in this study already contained 0.30% w/w sodium chloride. As the molar ratio of the salt to BSA is not negligibly small, this remaining salt was taken into account in calculating the sodium concentration. Here we used a new parameter, for convenience, to describe the amount of salt in sample solutions, instead of the actual sodium concentration. The molar ratio, here denoted as $R$, was defined as follows:

$$R = \frac{c_s + c_{sa}}{c_p} \tag{1}$$

Here $c_s$ is the concentration of sodium chloride contained in BSA, $c_{sa}$ the concentration of added salt, and $c_p$ the concentration of BSA. The units of these parameters are moles per gram of solution. The sample solutions were prepared in the range of $R$ from 9 (no salt added) to 100. The BSA concentration was adjusted from 2 to 20% w/w depending on $R$.

The effect of pH was examined for 10% w/w BSA solution in the pH range from 5.7 to 6.8. The pH was adjusted by adding 0.1 N hydrochloric acid or 0.1 N sodium hydroxide solution. (The use of buffer solutions was rejected as mixtures of ionic species would complicate the system even further.) Because lowering pH may produce aggregates, the acid was added very carefully. The amounts of acid and alkali added to sample solutions were so small that the ionic strength was thought to be unchanged.

For the binary gels, mixed solutions of two of the three proteins were prepared by dissolving each material in deionized water. The total concentration of proteins was adjusted to 10% w/w and the ratio of the two components was changed from 0:10 to 10:0 over five or six intervals. Since BSA has a lower native pH in solution (6.6) than the other two, the pH of the mixed solution was adjusted to 6.6 by adding 0.1 N hydrochloric acid. When acid was added, the solution sometimes became turbid or formed traces of coagulum but recovered with continuous stirring.

Details of the Carri-Med CSL100 instrument and procedures are given in Part 1. The frequency and the strain were set at 1 rad/s and 1%, respectively. While a sample was heated at a predetermined temperature, storage moduli $G'$ and $G''$ were monitored as a function of time. From the $G'$ trace we determined gelation time by means of the method adopted before. Measurements were performed at different temperatures from 50 to 90 °C.

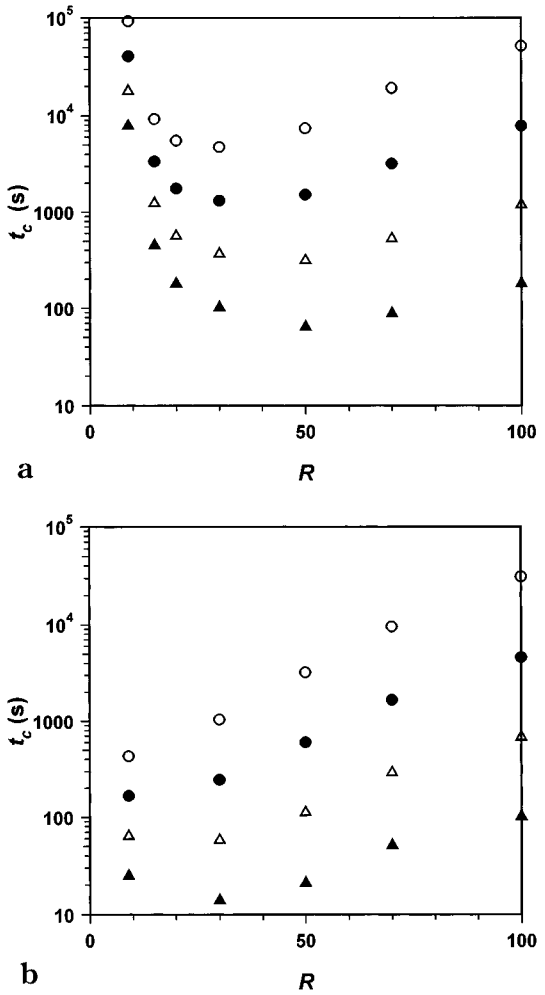


**Figure 2.** (a) Gelation time vs $R$ for BSA 8% w/w. Symbols correspond to different heating temperatures: 58 °C, open circles; 60 °C, filled circles; 62 °C, open triangle; 64 °C, filled triangle. (b) Same plot as in part a except for 15% w/w BSA.

## 3. Results

**3.1. Effect of Ionic Strength on BSA Gels.** The gelation time, $t_c$, for 8% w/w BSA solution containing sodium chloride was plotted against temperature, $T$, as shown in Figure 1. The symbols represent the value of $R$, the molar ratio of $Na^+$ to BSA. Here the symbol at $R$ equal to 9 corresponds to the sample without any added salt. This figure shows that by adding even a small amount of salt, $t_c$ became dramatically shorter. Since the slope changed slightly, the temperature dependence of $t_c$ was thought to be modified by salts. We can also observe a shift of the data at lower salt concentrations, but at higher salt there seems to be practically no effect, which may suggest that electrostatic interactions are screened at these salt concentrations. The data were fitted reasonably well by exponential functions of $T$. Figure 2a shows $t_c$ vs $R$ for the samples at various temperatures. It was found that $t_c$ did not change linearly but showed a pronounced minimum. The $R$ values at minimum $t_c$, $R_c$ seem to depend on $T$, with $R_c \sim 20$ at 58 °C and 50 at 64 °C. The figure also suggests that the initial drop was more significant at higher temperatures. The results at a

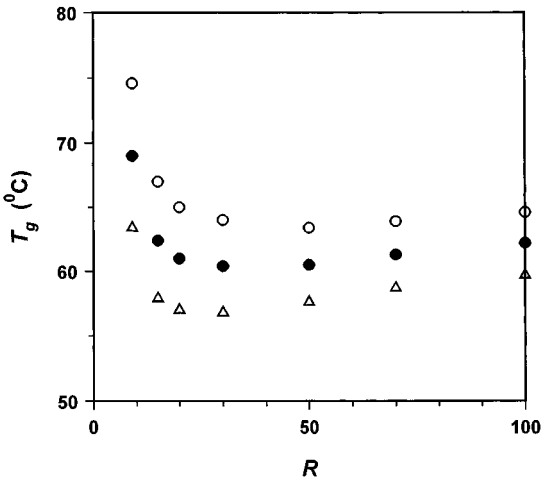


**Figure 3.** Gelation temperature vs $R$ for BSA 8% w/w. Symbols represent threshold times for gelation (open circles, 100 s; filled circles, 1000 s; triangle up, 10000 s).

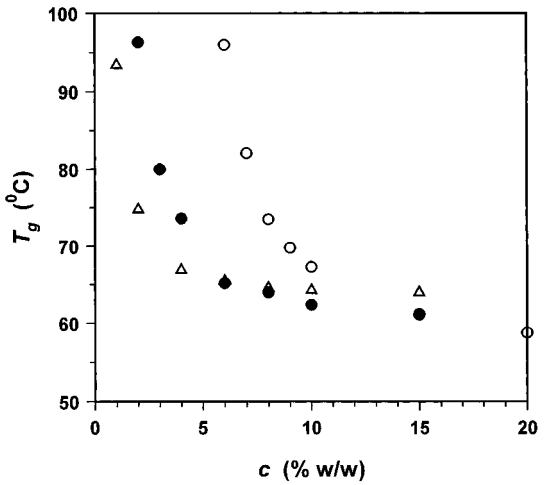


**Figure 4.** Gelation temperature vs BSA concentration. Threshold time: 100 s. Symbols correspond to different values of $R$: open circles, $R = 9$; filled circle, $R = 30$; open triangle, $R = 100$.

higher BSA concentration (15% w/w) are shown in Figure 2b. The general features seem similar to those for 8% w/w BSA, although the effect of salt becomes less significant as the BSA concentration increased, which may reflect the contribution of protein counterions to the charge screening.

From the data shown in Figure 1, we estimated the gelation temperature, $T_g$, by setting a threshold time for gel formation, in this case, 100, 1000, and 10000 s. The results are presented in Figure 3, in which $T_g$ is plotted against $R$. This shows that $T_g$ decreased rapidly on addition of a small amount of salt and increased slightly after the minimum point. The effect of salt seems rather different depending on the threshold time. This figure can be seen as a state diagram which predicts gelation conditions from $T$ and $R$. The regions above the points show that the sample is in the gel state, while those below are in the sol state. The boundaries between the two regions correspond to the gelation curve, but in this case a time effect is also involved.

In particular, the initial decrease in $T_g$ with the increase in $R$ becomes smaller. The same method can be applied to the $R$ dependence of $t_c$. The results are summarized in Figure 4. Here $T_g$ is plotted against BSA concentration, $c$, at various $R$ values. The data at $R$ equal to 9 were for the sample without addition of salts,

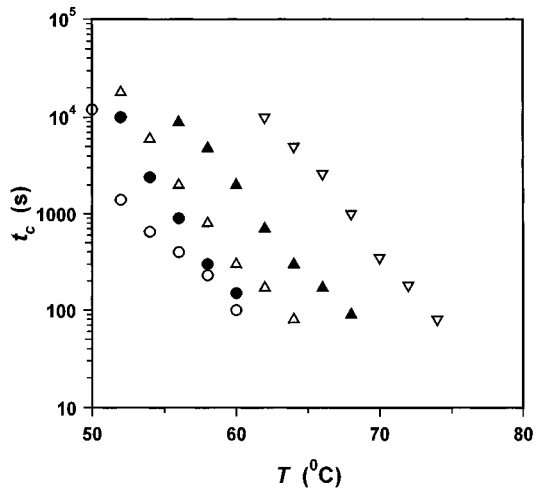


**Figure 5.** Gelation time vs temperature for BSA 10% w/w. Symbols correspond to different pHs: 5.7, open circles; 6.0, filled circles; 6.3, open triangle up; 6.6, filled triangle; 6.9, open triangle down.

and therefore they are the same as for the single component system given in Part 1. It is seen that addition of salts changed the gelation curve quite markedly; the curve shifted to lower BSA concentration as $R$ increased. As a result, the critical concentration for gel formation became very much smaller, i.e. ca. 1% w/w at $R = 100$. The other data for longer threshold times (1000, 10000 s) also showed a similar trend. Although previous measurements have suggested this effect, this is the first time the salt effect on the sol−gel boundary has been established unequivocally. The superimposition of data at higher BSA concentrations may reflect again the increased contribution of protein counterions to the charge screening process.

**3.2. Effect of pH.** In Figure 5, gelation time, $t_c$, for 10% w/w BSA solution is plotted against temperature, $T$. The pH of the samples ranged from 5.7 to 6.9 as shown in the legends. The figure shows that $t_c$ became appreciably shorter as pH was lowered. On this semilog scale, the data were reasonably linear in $T$ and their slopes were nearly the same, except for the sample at the lowest pH (5.7). When the same data were plotted against pH (not shown), It was found that $t_c$ decreased rapidly as pH became lower, and it seemed to approach a minimum around pH 5.5, although this was not confirmed because of experimental difficulties at lower pHs. In Figure 6, $T_g$ against pH is shown at different threshold times for gelation. The determination of $T_g$ was performed as previously described, and suggests that $T_g$ was lowered with the decrease in pH and approached a plateau value.

**3.3. BSA and $\beta$-Lg Mixed System.** Measurements on the $\beta$-Lg system were performed as for BSA (Part 1) and a corresponding state diagram (Figure 7) was constructed. In Figure 8, gelation time, $t_c$, is plotted against temperature, $T$, for the BSA/$\beta$-Lg mixed system at various ratios as shown in the legend. The $t_c$ was found to increase with the ratio of $\beta$-Lg. The data again seem to be linear on a semilog scale, although less clearly so for the three middle series (6:4, 5:5, 4:6). It was also seen that the slope of the curves became less steep as [$\beta$-Lg] increased. Although the change in $t_c$ was not perfectly linear for each series, we fitted them with exponential functions of $T$, and the corrected values of $t_c$ were used for the remaining figures.

The $t_c$ data were plotted against the ratio in weight of the two proteins in Figure 9. It is clearly shown that

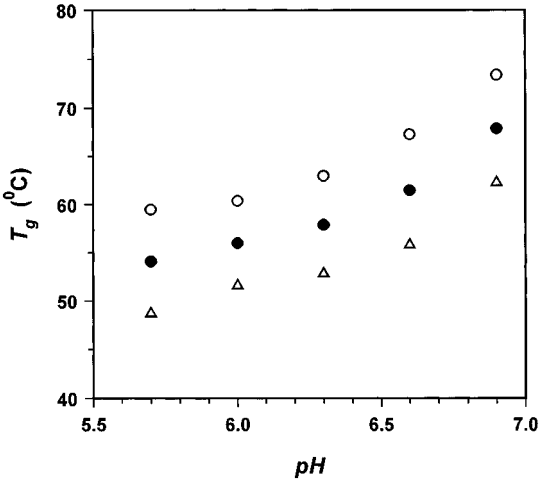


**Figure 6.** Gelation temperature vs pH for BSA 10% w/w. Symbols represent threshold times for gelation (open circles, 100 s; filled circles, 1000 s; triangle up, 10000 s).

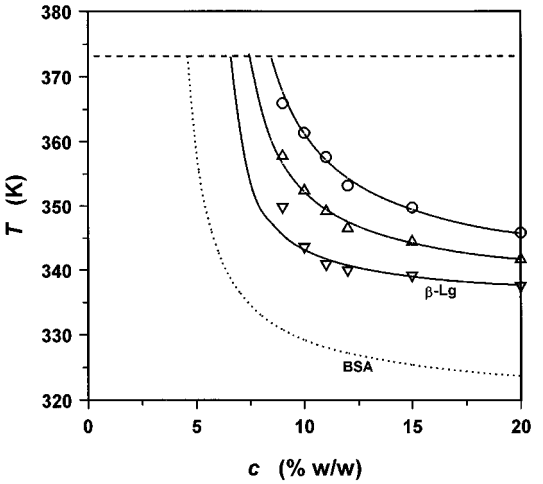


**Figure 7.** Temperature vs concentration state diagram for $\beta$-Lg illustrating the sol/gel boundary. Symbols represent threshold times for gelation (circles, 100 s; triangle up, 1000 s; triangle down, 10000 s); curves were obtained from eq A-13 of Part 1.[1] The horizontal dashed line indicates the boiling point of the aqueous solvent; the small dashed line represents the 1000 s threshold data for BSA.

the change in $t_c$ against the protein ratio could not be expressed by straight lines. Here, as the ratio of $\beta$-Lg rose, $t_c$ increased rapidly at first but gradually the change became less pronounced. This tendency was not very different for other temperature levels used in this experiment.

Figure 10 shows the gelation temperature, $T_g$, vs the protein ratio at various threshold times. It was found in this figure that $T_g$ became higher as $\beta$-Lg increased, but it did not rise linearly. The change in $T_g$ became smaller at longer threshold times. The theoretical curves drawn in both figures, are calculated as described in section 4.3.

**3.4. BSA/α-La and $\beta$-Lg/α-La Mixed Systems.** Gelation time, $t_c$, vs temperature, $T$, for BSA and α-La mixed systems is shown in Figure 11, while that for $\beta$-Lg and α-La system is given in Figure 12. As can be seen, these combinations did not give perfectly reproducible results. In particular, the series in which the α-La ratio was higher than the other showed rather large deviations. The main reason is most likely that these mixed proteins needed rather high temperatures (>90 °C) and relatively long measurement times (>1000 s) for gel

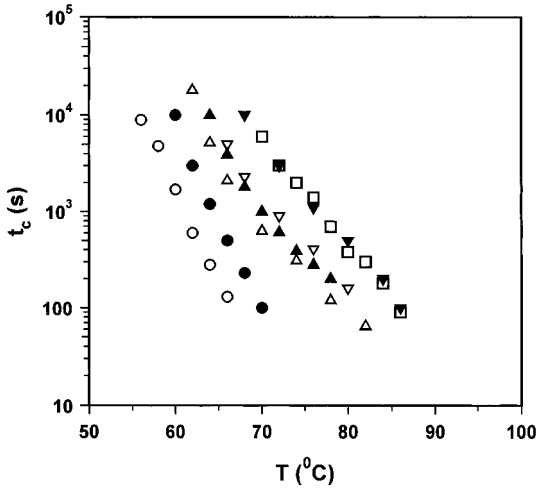


**Figure 8.** Gelation time vs temperature for the BSA/$\beta$-Lg mixed system. Symbols represent different ratios of BSA to $\beta$-Lg: 10:0, open circle; 8:2, filled circle; 6:4, open triangle up; 5:5, filled triangle up; 4:6, open triangle down; 2:8, filled triangle down; 0:10, open square.

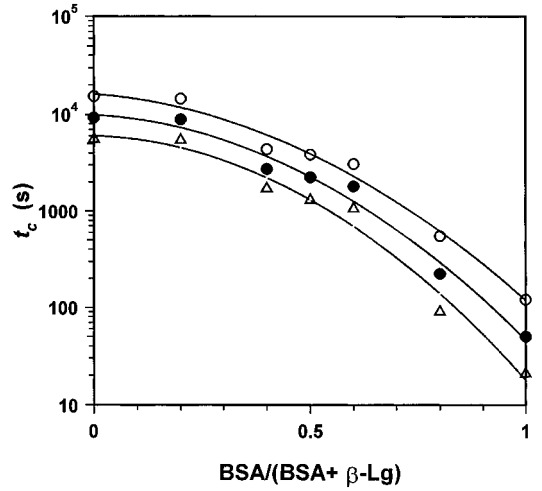


**Figure 9.** Gelation time vs the protein ratio for the BSA/$\beta$-Lg mixed system. Symbols correspond to different heating temperatures: 66 °C, open circles; 68 °C, filled circles; 70 °C, triangle. Theoretical lines were calculated from eq 2.

formation. It was probable that evaporation of water occurred and the protein in the solution was partially concentrated. This would cause the large deviations in the apparent $t_c$.

Although we tried measuring these systems several times, the quality of data did not seem to improve. We felt that the inherent problems would not be solved unless the instrument and consequent procedures were completely changed. One possibility would be to use a sealed pressure rheometer at different extreme pressures and extrapolate results to the vapor pressure of the protein system. However, these combinations were not examined any further in this work. We hope, however, to reexamine them in the future.

## 4. Discussion

**4.1. Effect of Ionic Strength.** These experiments revealed a pronounced effect of sodium chloride on the gelation time for BSA, the nature of which has not been reported in such detail before. Although the results seem rather complex, it is possible to draw several conclusions as follows:

1. The addition of sodium chloride changes the gelation time, $t_c$, dramatically. The strength of the effect depends on the BSA concentration and temperature.

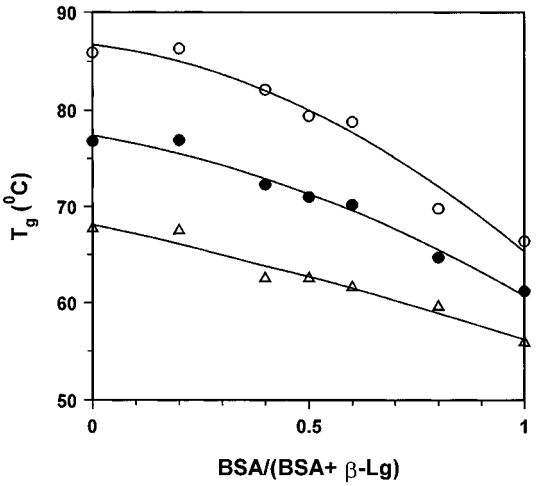


**Figure 10.** Gelation temperature vs the protein ratio for the BSA/$\beta$-Lg mixed system. Symbols represent threshold times for gelation (open circles, 100 s; filled circles, 1000 s; triangle up, 10000 s). Theoretical lines were calculated from the temperature analog of eq 2.

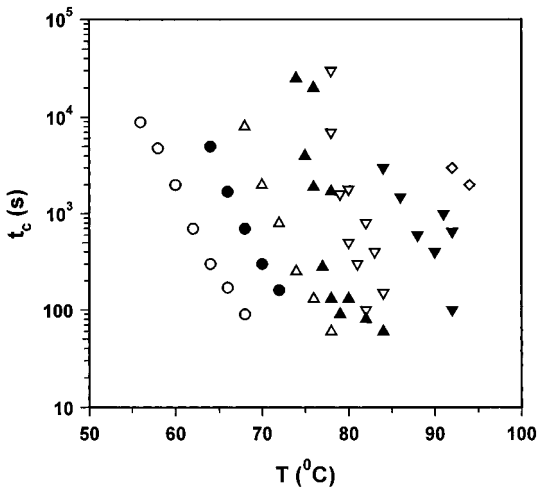


**Figure 11.** Gelation time vs temperature for BSA/α-La mixed system. Symbols represent different ratios of BSA to α-La: 10:0, open circle; 9:1, filled circle; 8:2, open triangle up; 6:4, filled triangle up; 4:6, open triangle down; 2:8, filled triangle down; 0:10, diamond.

2. At lower BSA concentrations, the effect of sodium chloride is more significant. Gelation time, $t_c$, decreases with salt addition and shows a minimum. Further addition makes $t_c$ longer again. At higher BSA concentrations, the initial decrease becomes less pronounced and $t_c$ increases monotonically.

3. At lower temperatures, $t_c$ shows a minimum at smaller $R$. As the temperature becomes higher, $R_c$ at the $t_c$ minimum shifts to larger values.

4. Gelation temperature $T_g$ decreases on salt addition and shows a minimum. Further addition makes $T_g$ longer again. At higher BSA concentrations, the initial decrease becomes less pronounced and $T_g$ increases monotonically.

5. At lower threshold times, $T_g$ shows a minimum at higher $R$. As the threshold time becomes higher, $R_c$ at the $t_c$ minimum becomes smaller and finally appears to vanish.

6. Critical concentrations become lower as $R$ increases.

Here we discuss the effect of salts on the basis of the widely accepted model, that protein gelation proceeds

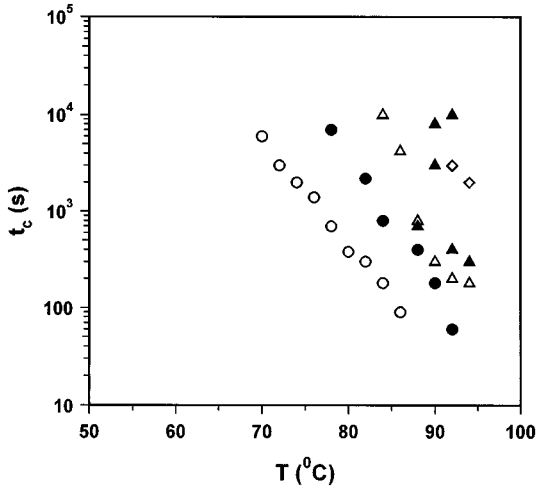


**Figure 12.** Gelation time vs temperature for the $\beta$-Lg/α-La mixed system. Symbols represent different ratios of $\beta$-Lg to α-La: 10:0, open circle; 8:2, filled circle; 6:4, open triangle up; 2:8, filled triangle up; 0:10, diamond.

in two steps, denaturation of native proteins and subsequent aggregation.

There have been a number of studies on electrostatic contributions to protein folding, because protein denaturation is usually the result of unfolding. Salts were once believed to affect electrostatic shielding around the protein.[6] This was explained by the balance of classical electrostatic effects and specific charge interactions. Increasing charge repulsion can destabilize the folded protein, because the charge density on the folded molecule is greater than on the unfolded molecule. The latter is exemplified by ion pairing (salt bridging), which will stabilize the folded proteins. As these two interactions are balanced, addition of salts can stabilize proteins if the classical effects are dominant but destabilize them if specific interactions are important.

However, the idea now accepted[6] is that the electrostatic interactions are not sufficient to explain protein folding, and instead the hydrophobic interaction must be the most important interaction. These principles are applicable to protein denaturation, so the electrostatic interaction around the protein particles is expected to change with salts. It is likely that addition of electrolytes will cause some ion pairing and will suppress the denaturation caused by heating. Consequently, it may be said that salts delay the denaturation process. The work by Renard and Lefebvre[7] on heat-set $\beta$-lactoglobulin gels is important here, since except close to the isoelectric point they have shown that addition of salt lowers the critical gel concentration, $C_0$, significantly. By direct analogy with their model, the same factors should lower $t_c$. Essentially the same effect was seen by Yamasaki and co-workers[8,9] in their investigations of the effect of pH and ionic strength on the denaturation temperature by DSC. Here we may appeal to basic lyotropic effects, that is to say the influence of salts on "water structuring", and the consequent alteration of hydrophobic stability. Overall it could be argued that aggregation of denatured proteins species will tend to be encouraged by higher salt (just by charge suppression) and protein concentration counterion effects and discouraged by the formation of intramolecular salt bridges and by general lyotropic effects. Gelation time is considered to reflect the overall reaction rate of both denaturation and aggregation processes. Both the initial decrease and subsequent increase in $t_c$ seen in

Figure 2, parts a and b, may be explained, although only in qualitative terms. This discussion is necessarily speculative, and needs further experimental support, but essentially follows that in refs 7−9.

There are few viscoelastic measurements on the salt effect on gelation points, although the work by Renard and Lefebvre[7] is again very relevant here. A paper by Nyström and co-workers[10] has examined the gelation of ethyl(hydroxyethyl)cellulose in the presence of salt and surfactant. However, there are more papers dealing with fully cured gels with salts as reviewed by Clark and Lee-Tuffnell,[11] and Clark and Ross-Murphy.[12] Such studies have been carried out with various experimental techniques such as rheometry, X-ray or light scattering, and electron microscopy. One important observation from these studies is that resulting gels become rather turbid according to the salt concentration. For example, Richardson and Ross-Murphy[13] compared the optical appearance of BSA gels with X-ray scattering data and determined a form of phase diagram which shows three different state of gels as a function of salt concentration and pH: clear gel, turbid gel, and opaque coagulate.

Microscopic observation was also carried out by Stading and Hermansson.[14−16] They found that the microstructure of $\beta$-lactoglobulin gels changes dramatically by adding salts and tried categorizing into the two groups: fine stranded gels (homogeneous) and particulate gels (heterogeneous). We can find similar results in many other papers, but the factors which determine the structure do not seem to be fully understood. Furthermore, as the measurement of the cured gels are not as sensitive as that of the gelation point, it is very difficult to analyze the results in a quantitative way. Indeed a further rationale for the present measurements is that most mechanical properties of gels can be related back to the critical behavior.

In our experiments, the change in appearance of samples was observed as well. It was found that the samples became rather turbid at high salt concentrations and temperatures, even before gel formation, although these changes did not affect gelation time significantly. It is likely that addition of salts decreased the solvent quality and made the system more heterogeneous. A drastic change in gelation behavior on addition of salt was found in our rheological experiments, but it would be very worthwhile to examine the possible structural changes of the protein during the gelation process. The comparison of rheology with structure must be able to give us important information on protein gelation in the presence of salts.

**4.2. Effect of pH.** The pH dependence of gelation time can also be discussed in terms of electrostatic effects. As shown earlier, decreasing pH was found to make the gelation time shorter. This seems to be a result of the neutralization of charges on protein particles. Since the pH of experiments (6.6) was higher than the isoelectric point (5.1), the protein should be stabilized by negative charges. If the pH is shifted to the isoelectric point, the protein particle becomes less charged, and therefore its stability is lowered. As a result, denaturation and/or aggregation are promoted more strongly and gelation time becomes shorter. Again, this cannot be confirmed at this stage, and more detailed data are necessary, in particular, structural information.

In contrast to the very few studies on incipient gelation behavior, the pH effect on fully cured protein gels were reported by some workers. As already mentioned, Richardson and Ross-Murphy,[13] and Stading and Hermansson[14−16] have examined this problem. The results seem to be analyzed successfully in terms of homogeneity/heterogeneity of gel structure and can also be displayed in diagrams. Although the detailed mechanism is still unclear, at least a qualitative description seems established. Any further studies to be made concerning the pH effect would help to explain the structural change caused by pH change and connect it to the incipient behavior of protein gelation, which is also strongly dependent on pH.

It should be noted, however, that there is a problem in preparing samples. To adjust the pH of the protein solutions, it is necessary to use acid or alkali. This of course may change the ionic strength of the solution, particularly when the pH change is large. As we have already shown in this chapter, protein gelation is very sensitive to the ionic strength, and therefore such effects also have to be taken into account. Most of the studies previously performed paid rather scant attention to this point, probably because the experiments were not sensitive enough. In any study of the pH effect on gelation points, the concomitant change in ionic strength is, without doubt, an important factor to be considered.

**4.3. Mixed-Protein Gel Systems.** As the only truly reliable data obtained in this study were for the BSA and $\beta$-Lg mixed system, we limit our discussion to this combination. By comparison with $t_c$ and $T_g$ for single component systems, it may be said that the results for this two-protein gel system were not completely independent of each other, but they are compatible to a certain degree. This is because if the two components did *not* interact with each other but formed gels individually, gelation time and temperature could only be measured in the high BSA or high $\beta$-Lg region, i.e. when the protein concentration ratio is $<0.1$ or $>0.6$. If the ratio between them is, e.g., 0.5, the concentration of neither component is high enough for gel formation. (We can neglect here the effect of physical crowding since the overlap concentration, $c^*$, ∼30% as discussed in Part 1). However, gelation was clearly observed even in this intermediate range.

These results suggest that the binary protein solutions may have formed mixed biopolymer gels. According to Brownsey and Morris,[17] such mixed gels can be subdivided into interpenetrating, phase-separated, and coupled networks. For our globular protein mixtures, the compatibility of the components would probably be good enough to maintain some compatibility. Hence, it is likely that the mixed proteins formed interpenetrating networks or coupled networks but not phase-separated networks. If "cross-links" were formed between polymer chains of the different species as well as of the same species, the resulting gels would be coupled networks. If not, the interpenetrating networks must be involved. Although clear evidence for identifying which network is formed has not been obtained, we feel that the protein mixtures used in this study may have formed coupled networks. This is because a protein molecule, once denatured, will probably lose any aggregation specificity. To confirm this, it is necessary to take more structural information obtained by electron microscopy or scattering measurements. Unfortunately the mutual contrast would most likely be very low, so special techniques, for example, by raising immunofluorescent stains for one or other of the proteins, would be needed to visualize this protein in the presence of the other within the gel system. Another useful ap-

**Table 1. Coefficients of Equation 3**

| $T$ | $a_1$ | $a_2$ | $a_3$ |
|---|---|---|---|
| 66 | 4.77 | 9.69 | 4.17 |
| 68 | 3.83 | 9.19 | 4.85 |
| 70 | 2.88 | 8.70 | 5.54 |

**Table 2. Coefficients of Equation 4**

| $t$ | $b_1$ | $b_2$ | $b_3$ |
|---|---|---|---|
| 100 | 65.2 | 86.7 | 16.1 |
| 1000 | 60.7 | 77.4 | 90.5 |
| 10000 | 56.2 | 68.1 | 20.4 |

proach may be FTIR microscopy, as used by Durrani and co-workers.[18,19]

Although it would be reasonable to assume that binary protein mixtures formed a kind of mixed gel, it is not easy to explain the experimental data for such mixed gels. In Figure 8, $t_c$ does not change as a linear function of protein ratio. However, as demonstrated earlier for single component systems, $t_c$ can be regarded as a linear parameter after logarithmic conversion. Therefore, it would be justifiable to use $\ln(t_c)$ to discuss apparently linear behavior. In these figures it is seen that $t_c$ cannot be expressed as a simple lever rule of the ratio of the components, but instead, it shows a certain curvature. This suggests that we should take account of the cross-term effect in analyzing the results.

Here we attempt to fit the results with simple empirical formulas to encompass the behavior. First, gelation time $t_c$ is assumed to be expressed by a linear relation (after logarithmic conversion) such as

$$\ln t_c = r \ln t_{c(\mathrm{BSA})} + (1 - r) \ln t_{c(\beta-\mathrm{Lg})} \qquad (2)$$

where $t_{c(\mathrm{BSA})}$ and $t_{c(\beta-\mathrm{Lg})}$ are the gelation times for each pure component and $r$ is the weight ratio of BSA relative to the total protein, i.e. BSA/(BSA + $\beta$-Lg). This is the simplest form, which is illustrated as a straight line on a semilog scale and should work if there is no significant cross-term effect. However, as mentioned above, the data did show deviations from linearity.

Hence, we introduce a new term to account for the interaction between the proteins. Then the above equation becomes

$$\ln t_c = a_1 r + a_2(1 - r) + a_3 r(1 - r) \qquad (3)$$

where $a_1$, $a_2$, and $a_3$ are coefficients, and the third term represents the cross-term effect. Here the gelation time for each component $t_{c(\mathrm{BSA})}$ and $t_{c(\beta-\mathrm{Lg})}$ are altered by the coefficients, because they include experimental errors and are not necessarily fixed. Obviously, if the fitting is made well, the estimated values of $a_1$ and $a_2$ should not be so far from the $t_{c(\mathrm{BSA})}$ and $t_{c(\beta-\mathrm{Lg})}$.

For the relationship between gelation temperature $T_g$ and the protein ratio $r$, an exactly analogous procedure was employed, with respective coefficients $b_1$, $b_2$, and $b_3$. For the same reason, the apparent gelation temperature for each component is modified when fitting $b_1$ and $b_2$. Here gelation time $t_c$ was converted to the natural logarithmic form, but gelation temperature $T_g$ was not.

$$T_g = b_1 r + b_2(1 - r) + b_3 r(1 - r) \qquad (4)$$

These models were applied to the data and coefficients of fitting curves were determined. The fitting curves are shown in Figures 9 and 10, and the respective coefficients are given in Tables 1 and 2. It can be said that the fitting was reasonably successful, and the resultant regression coefficients were quite satisfactory. More details are given elsewhere.[20] The determined values of $a_1$ and $a_2$ correspond to $\ln t_c$ for BSA and $\beta$-Lg, while $b_1$ and $b_2$ correspond quite closely to the respective $T_g$s. Although these were "floated", the results were very close to the original values.

As the models shown here were constructed in a purely empirical way, we cannot discuss the further details of gelation mechanism for protein mixed systems. Nevertheless, at least some information can be drawn from the results. For example, the coefficient $a_3$, which represents the contribution of the interaction between the proteins, was found to increase with the rise in temperature. This suggests that the compatibility of the components became better at higher temperatures. The corresponding gelation temperature coefficient implies that the effect of the interaction between the proteins became less pronounced at longer measurement times. This could be explained as a reorganization of the structure within the gel. As a result, the effect of the interaction became smaller. At this stage, this is merely a speculative interpretation, but such an effect can be successfully expressed in a quantitative way. We can expect that this kind of approach may give some information about mixed systems.

It may also be possible to explain these results by means of the model for single systems which we proposed in Part 1. In the model, the parameter, $N_{b0}$, which determines the gelation point as described in eq A-1 of that paper, is responsible for describing the gelation process. If this variable is modified for binary systems, gelation time may be expressed by the use of the parameters in binary system, e.g. concentration ratio and apparent reaction orders.

At this stage, however, we have not yet found an appropriate solution for its application to binary systems. The treatment of such formulas is obviously very complicated, and without certain approximations, it is almost impossible to develop equations. Even if these were to be developed, the number of undetermined (or overdetermined!) parameters would seem to be a continuing problem. Nevertheless, we hope to continue the analysis in future work.

## 5. Conclusions

The effects of environmental factors, salts and pH, on protein gelation, were investigated with the use of rheological techniques. In particular the gelation time was significantly influenced by these factors, a result which has not been reported in detail before. Moreover, no detailed analysis of the data has been carried out to date, because the behavior is very complex. It is hoped that further experiments and theoretical analysis will enable us to explain the protein gelation under various conditions.

The heat-induced gelation of binary globular protein mixtures was investigated by the same techniques. Unfortunately we have obtained reliable results only for the BSA and $\beta$-Lg system because of the difficulty in experiments. This problem needs to be solved by adapting the instrument and procedure for measurement. From the data of the BSA/$\beta$-Lg system, it was found that gelation behavior changed significantly according to the ratio of the two proteins. The change in the gelation time and temperatures were not expressed

as a linear equation but required a cross-term. Although we feel that detailed mechanisms of gel formation of protein mixtures have not been revealed here, the present approach may give us some clue to the studies of such complex systems. We hope that the further investigation will be carried out by expanding our method and also by employing other experimental techniques.

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