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A study by photon correlation spectroscopy of the influence of gelatin source, ionic strength and temperature on the gelation process

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Summary

Photon correlation spectroscopy was used to study the decrease in diffusion coefficient of the gelatin molecule during the early stages of gelation. The rate of gelation was found to depend on gelatin source, but the kinetic pathway appeared similar for the six samples observed. In 6% w/v polymer solutions, gelation was not significantly affected by the ionic composition of the medium. However, over a narrow range of 1.4°C the rate of gelation appeared extremely temperature dependent. The rate constants k (s^{-1}) obtained from the plots of log D vs time displayed a linear relationship when expressed by a modified Arrhenius equation of log k (s^{-1}) ~ $1/T\Delta T^{\circ}$ (K^{-2}). An activation energy of 210 kJ mol^{-1}, calculated from the gradient, was of a similar value to that quoted by Harrington and Rao (*Biochemistry*, 9 (1970) 3714–3733).

Introduction

The term 'gelation' embraces a series of events which comprise the gelatin setting process. These are generally summarised as pregelation, setting and maturation but whilst the precise nature of the process remains speculative, existing theories display common salient features. In aqueous solutions above 35–40°C, the polypeptide chains are molecularly dispersed and adopt a random coil conformation. At lower temperatures the viscosity of the solution increases due to the preferential linking of some of the gelatin molecules to form aggregates and is regarded as the pregelation stage. Above concentrations of 0.5% a gel results and the solution is termed 'set'. 'Setting' is claimed to be the result of a partial return of the disordered molecules to the parent collagen, a triple helix. The formation of the triple helix and preceding events are important in determining the kinetics of gelation and identifying the rate-controlling step. During the maturation phase a subsequent increase in order of the molecules, stiffening of the junctions and a general thickening of the gel network is observed.

Gelation and the characteristics of the resulting gel appear to be a fine balance of many interacting factors. The structure of a gel network

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is determined by its mode of formation and hence its thermal history. Snap chilling or high polymer concentration, produces a 'fine' network. Under these conditions, the molecules are frozen in their disordered state and linking is by random contact leading to many weak bonds. Slow cooling results in a 'coarse' network, typical of many dilute gels, where only the strongest bonds are able to survive the higher temperatures. Consequently, linkages are ordered and continue to strengthen as a result of restricted thermal motion.

Although polymer concentration influences the degree of molecular interaction and the subsequent linkages formed during gelation, it is also claimed that the higher the gelatin concentration, the less effect solvent environment has on the setting process. In this respect, ionic composition would be considered less important in more concentrated polymer solutions. The setting process is also influenced by the nature and source of the gelatin, since multi-chain gelatins have been shown to increase rates of helix regeneration compared to their corresponding single chains (McBride and Harrington, 1967). Harrington and Rao (1970) found that α_1-chains have a greater concentration dependence than α_2-chains in the renaturation process, thus suggesting that the varying proportions of component species, i.e., α-, β- and γ-chains in commercial gelatins, may be inherent to their gelling properties.

The above parameters are difficult to study in isolation and therefore require a detection system which adequately monitors the combined effects. The salient features of setting, i.e., aggregation, configurational changes and decrease in chain mobility, predictably influence the light scattering properties of the system. In the following work, photon correlation spectroscopy was used to translate this information, and thus enable the study of the diffusing gelatin molecule over the early stages of gelation. An attempt was made to relate photon correlation spectroscopy (PCS) to stages in the setting process. The effects of gelatin source on gelation and on the kinetics of the setting process are illustrated using four alkaline- and two acid-processed gelatins. The sensitivity of gelation to ionic strength and to fluctuations in temperature was demonstrated using a fixed gelatin concentration. The study outlines how rate constants can be determined from this work and utilized in a modified Arrhenius equation to determine activation energy associated with the gelation process.

Materials and Methods

The following limed ossein gelatins were used: Rousselot 32272, Rousselot 32976, Croda 174 and Neinburger 06528; the acid ossein gelatins were: Rousselot 05759 and Croda 163.

6% w/v gelatin solutions were prepared using Sorenson's buffer (pH 7, ionic strength 0.148) and held at 50°C for 2 h to destroy thermal history. At the end of this period, the solutions were quenched to 26 ± 0.1°C, by placing in the temperature-controlled vat of the spectrophotometer (Malvern Instruments, Malvern). Light scattering experiments were performed immediately at a sample time of 500 μs over an experimental duration of 100 s. Diffusion coefficients were determined every 2.5 min and the data analysed by single-exponential analysis (Malvern Application program [k7025-spec-22]). The effect of ionic strength on gelation rates was shown by adjusting 6% w/v solutions of Rousselot 322272 limed and 05759 acid samples to pH 7 with HCl or KOH (ionic strength $\sim 10^{-7}$) and studied as above.

The temperature dependence was determined using 6% w/v solutions of Rousselot 32272 limed ossein gelatin prepared and treated as above. The decrease in the slow diffusion coefficient was monitored at temperatures of 25, 25.5, 26 and 26.4°C. The data were analysed by single-exponential analysis using a sample time of 500 μs and experimental duration of 100 s.

Results

Figs 1 and 2 illustrate the linear relationship obtained between log D vs time for the six (6% w/v) gelatin solutions analysed by single-exponential analysis (26°C; sample time 500 μs). The rate constants determined from the plots are displayed in Table 1. Table 2 shows that although

TABLE 1

Gelation rate constants determined at 26°C for six gelatins from various sources

6% w/v gelatin solutions	k ($\times 10^{-2}$) (s^{-1})
Rousselot limed ossein 32272	0.200
Rousselot limed ossein 32976	0.339
Croda limed ossein 174	0.217
Neinberger limed ossein 06528	0.100
Rousselot acid ossein 05759	0.400
Croda acid ossein 163	0.511

a greater ionic strength increases the gelation rate of an ossein or acid processed gelatin, this is not a marked effect.

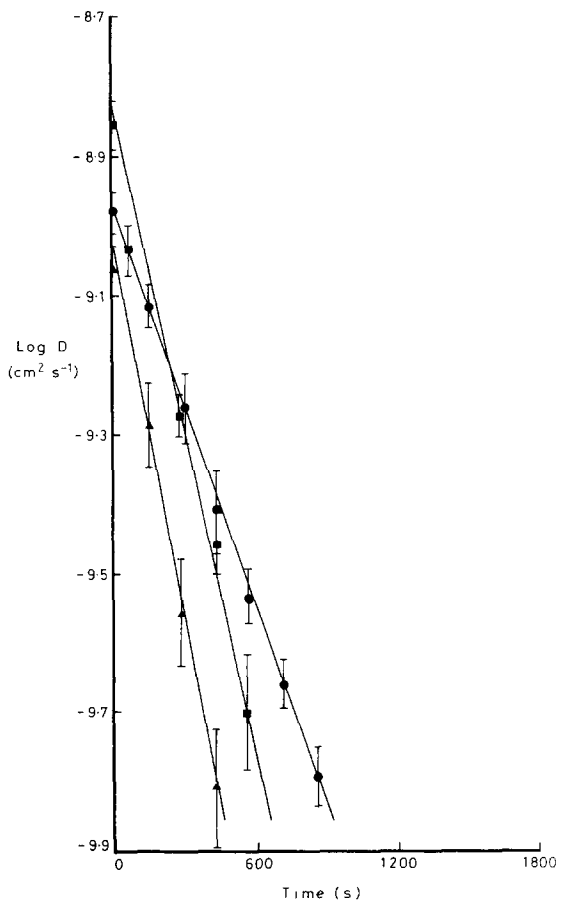

Fig. 1. Log D vs time for 6% w/v gelatin solutions at pH 7 and 26°C: Rousselot 32976 (■), Croda 174 (●) and Rousselot 05759 (▲).

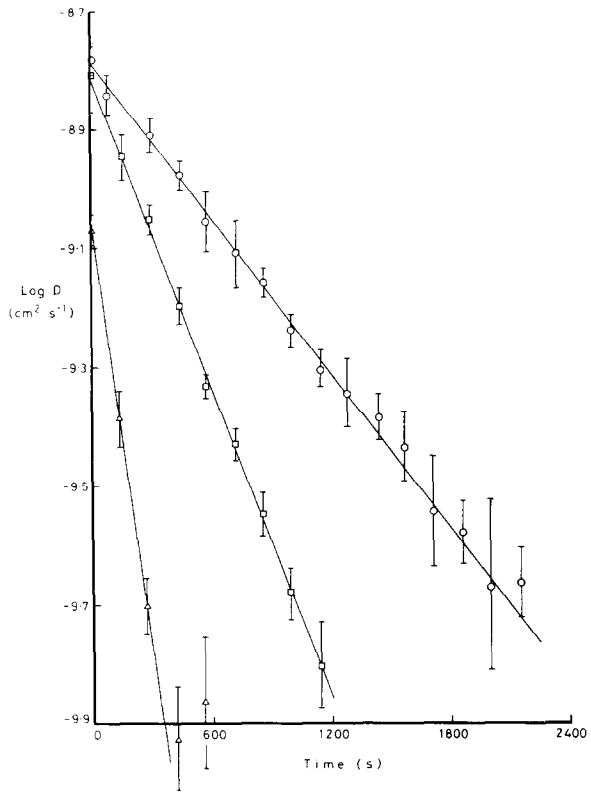

Fig. 2. Log D vs time for 6% w/v gelatin solutions at pH 7 and 26°C: Rousselot 32272 (□), Neinberger 06528 (○) and Croda 163 (△).

The rate of gelation was found to be extremely sensitive to temperature variation over the narrow range of 1.4°C (Fig. 3). Table 3 lists the rate constants determined from these plots. Fig. 4 illustrates the linear relationship obtained for a modified Arrhenius plot of log K (s^{-1}) vs $1/T\Delta T°$ (K^{-2}) where ΔT equals the degree of undercool-

TABLE 2

The influence of ionic strength on the gelation rate constant for a limed and an acid ossein gelatin

6% w/v gelatin solutions	Ionic strength $\sim 10^{-7}$ $k \times 10^{-2}$ s^{-1}	Ionic strength $= 0.148$ $k \times 10^{-2}$ s^{-1}
Rousselot limed ossein 32272	0.143	0.200
Rousselot acid ossein 05759	0.330	0.400

TABLE 3

The influence of temperature on the gelation rate constant for Rousselot 32272 limed ossein gelatin (6% w/v)

Temperature (°C)	k ($\times 10^{-2}$) (s^{-1})
25.0	0.468
25.5	0.315
26.0	0.200
26.4	0.125

ing, i.e., $(T_m - T)$ in K and T_m is the transition or melting temperature of the collagen structures (Flory and Weaver, 1960). An activation energy

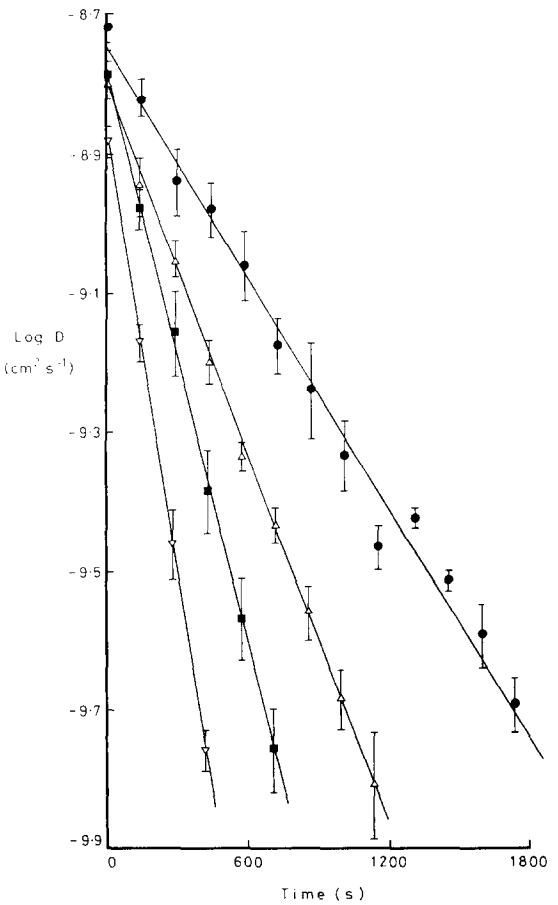

Fig. 3. Log D vs time for a 6% w/v gelatin solution (Rousselot 32272) at temperatures of 25°C (▽), 25.5°C (■), 26°C (△) and 26.4°C (●).

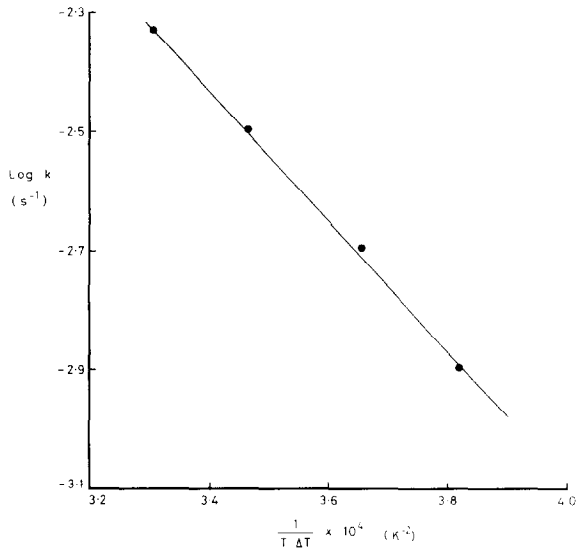

Fig. 4. Gelation rate constants expressed by a modified Arrhenius plot ($r = 0.999$).

of 209 790 J mol^{-1} was calculated from the slope using the following equation:

$$\log k = \log A - E_A/RT\ \Delta T(2.303)$$

where A is a constant, R represents the gas constant (8.31 J K^{-1} mol^{-1}), the slope $= -E_A/R$ (2.303) and E_A denotes the activation energy (J mol^{-1}).

Discussion

The light scattering phenomenon of the gelatin molecule is principally displayed as two decays, a 'fast' and a 'slow' mode (Thomas et al., 1985). The fast decay has been associated with a 'mutual' diffusion coefficient which expresses the internal motions or flexing of overlapping polymer chains. At infinite dilution the slow mode has been attributed to the slow relaxation of the self-diffusion coefficient. However, in more concentrated systems, where chain interaction is anticipated, the slow mode has been associated with the translation of the entangled polymer (Brown, 1983). The concentration dependence of the latter is claimed to parallel that of self-diffusion. In

PCS studies the two modes are separated by using suitable sample times.

The linear relationship obtained between log D (D representing the diffusion coefficient of the slow mode) vs time suggests that the data can be fitted to a single-exponential decay. The gradient, $d(\log D)/dt$, gives an indication of the rate at which the gelation process is taking place. However, as found by Eagland et al. (1974), this can only be pseudo-first order, since the rate depends upon the initial concentration and is thus first order only with respect to gelatin. It should be emphasised that the rate constants determined have no physical significance outside the scope of the work and are used as a comparative illustration only. Towards the end of the time scale a departure from linearity was observed. The reason for this is not accurately identifiable, since it may be due to combined effects, e.g., the formation of a network can give rise to heterodyning which demands a different detection system. Patterson et al. (1981) found that gelation led to inhomogeneities in their sample and claimed that the data were partially heterodyned by the scattering effects. These workers overcame the problem by stopping the reaction prior to the formation of the polygel. Therefore, to avoid possible complexities and resulting errors, only the pregelation phase was studied in the above experiments, which is to the point where flow of the gelatin solutions ceases.

In order to relate PCS to stages in the gelation process, mechanisms describing the reversion processes must be identified. Most of the studies in the literature do not entail gelatin gelation but the renaturation to the collagen structure. These studies have been on highly dilute solutions which are incapable of forming a gel, however, it is likely that gelation follows a similar sequence of events. Josse and Harrington (1964) proposed that each link involves the imino-rich section from three polypeptide chains. These develop from areas called primary nuclei which arise on each chain and consist of locked contiguous pairs of pyrrolidine residues. The primary nuclei incorporate adjacent triplets of similar stability, producing a single helix which is regarded as an unstable intermediate. The remainder of the chain is disordered, but growth on existing links during cooling decreases the distance between the links. As a result, hydrogen bonding between the chains increases, giving rise to a more stable triple-helical structure. Although the main event of renaturation and its role in gelation are agreed upon, controversy over the kinetics remains. Von Hippel and Harrington (1959) proposed the rate-determining step to be the aggregation of the single helices into the triple-helical structure, whereas Flory and Weaver (1960) regarded the single helix generated from the nucleated regions as determining the reaction rate. The instability of this species would mean that the aggregation of the single helices into the collagen structure would be accomplished very quickly. The process was regarded as first order, which appears to contradict the involvement of three chains in the proposed model. However, this was justified by the claim that the rate-determining step depended on the formation of a unimolecular species, i.e., the intermediate. Therefore, the process was first order with respect to gelatin concentration. Eagland et al. (1974) deduced that reversion consisted of two stages, an initial fast process which occurred over the first 20 min, followed by a slow process on a similar time scale. These workers determined the initial fast process as pseudo-first order with respect to gelatin, whereas the slow process was genuine first order. In later years variations on these theories have arisen and may be summarised as follows: below 0.1% and for single-chain gelatins, the renaturation process is intramolecular and summarised as first order. For solutions of higher concentrations which are capable of gelling, intermolecular reactions influence the process and may result in higher orders.

In the above work, the dramatic decrease in the diffusion coefficient observed over the time period may equate with the fast step in gelation. During the initial 'nucleation', areas of the chain adopt a poly(L-proline)II configuration. The 'locking in' of these helical sections results in a general stiffening of the chain and an increase in viscosity is observed. Whether growth of the helical structure over the length of the chain is also occurring during this period depends on which is the rate-determining step. Some workers, e.g.,

Josse and Harrington (1964) believe that adjacent pyrrolidine residues remain 'locked' during the collagen > gelatin transition, resulting in residual helical structure and thus obviating the need for a nucleation step. This would suggest that the formation of a single-helical chain is reflected in the above work. However, no gross misfit in the correlation function was observed during this period, indicating the lack of structure during the time course. Assembly of 'triple helices' and cross-linking effects may possibly be thought of as giving rise to inhomogeneities which would have been reflected in the autocorrelation function and resulting diffusion coefficients. This substantiates the assumption that helical formation may be the cause of the departure from linearity noted at the end of the study period.

The six gelatins demonstrated similar gelation profiles when studied under similar setting conditions. Thus, it appears that gelatins from varying sources follow similar pathways to reach the gel from the sol state. However, the difference observed in gelation rates suggests that the nature of the gelatin is influential in determining the kinetics of the gelling system. In contrast, Table 1 demonstrates that at 6% w/v polymer concentration, ionic strength is not important in determining the rate of gelation.

Fig. 3 illustrates the temperature sensitivity of the gelation process which is claimed to be typical of polymer networks which are stabilised by secondary forces (Stainsby, 1977). Formation of the network involves a delicate balance between polymer-polymer and polymer-solvent interactions, the balance of which is strongly dependent on temperature and on the microenvironment. The narrow temperature range restricts the use of an Arrhenius plot to determine activation energy. Extension of the range met with problems, since lower temperatures caused gelation to occur at almost immeasurably fast rates, whereas higher temperatures impracticably prolonged the process. Flory and Weaver (1960) found that an Arrhenius plot was non-linear, because the activation energy increases with temperature. They stated that in certain polymer systems this was consistent with the behaviour of the temperature coefficient of nucleated crystallisation processes. Similarly, in the above work, an Arrhenius plot was found to be non-linear but the modified plot of Flory and Weaver (1960) and, later, that of Harrington and Rao (1970), did show a linear relationship. The rate constants determined over the narrow temperature range (Table 3) bore a linear relationship in a modified Arrhenius plot (Flory and Weaver, 1960). For the calculation the transition temperature of collagen was taken as 35°C and the activation energy of 210 kJ mol^{-1} estimated from the slope was of a similar order to that determined by Harrington and Rao (1970) (in the above work, a linear relationship was also demonstrated between the rate constants vs T° (K) or $1/T$ (K^{-1}), but is of uncertain significance). Flory and Weaver (1960) noted a 1.6-fold increase in rate per degree between 20 and 23°C for gelatin solutions 0.06–0.4%. The above studies approximate to a 2.6-fold increase in rate per degree for 6% w/v solutions, which tends to suggest that the rate of reversion is more sensitive to differences in temperature than concentration.

Conclusion

Photon correlation spectroscopy has been used to study gelation and to determine the effects of polymer source, ionic strength and temperature on the setting process.

Earlier workers reported studies which mainly employed dilute systems which were incapable of gelling. However, because of the implications of molecular interaction, renaturation mechanisms operating at low concentration levels cannot always be extrapolated throughout the concentration range. Conversely, in concentrated solutions, it is difficult to isolate a course of events. In this respect, the above work has proposed to compromise these difficulties and has attempted to identify steps in the setting pathway. PCS can be regarded as a latter-day tool for studying gelation, since it appears that limited information has been obtained to date from traditional detection techniques such as polarimetry. The sensitivity of gelation to temperature as studied by PCS correlated with the results obtained by Harrington and

Rao (1970). This may further indicate the use of PCS in concentrated gelatin solutions as a valid detection system.

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