Influence of thermal treatments on the structure and stability of gelatin gels

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The influence of thermal history on the structure of gelatin gels has been investigated by measuring the specific optical rotation $[\alpha]_\lambda$ at $\lambda = 436$ nm. The helix content χ in the gels was derived by reference to the native collagen. Two types of thermal treatments have been applied: (a) cooling and heating at constant rates, and (b) quenching and annealing for long periods. Our results support the idea that the gels are non-equilibrium systems and exhibit thermal hysteresis. The kinetics of helix formation were analysed by the Avrami theory and compared to other techniques. The stability of the structures formed suggests the existence of several mechanisms of hydrogen bonding of the helices: disordered aggregation or partial collagen renaturation.

Keywords Gelatin gels; thermal treatments; stability

INTRODUCTION

Gelatin is a protein which has the noticeable property of forming elastic gels at room temperature for relatively low concentrations (a few per cent of gelatin in water).

A great deal of experimental work has been done related to the practical uses of gelatin[1] and to the fundamental aspects implied in the gelation process[2]. The conformational changes of collagen solutions, the sol–gel transition, the rheological properties of gels are still under investigation[1-4].

The gelatin gel is a physical thermoreversible gel: when a solution of concentration greater than 1% w/v is cooled below 40°C, a sol–gel transition occurs which starts by a progressive increase of the viscosity and the appearance of elasticity. If the temperature is raised, the gel 'melts', becoming liquid again. Some other synthetic or natural polymers (poly(vinyl alcohol), agarose,...) show a similar behaviour when dissolved in appropriate solvents and are generally termed 'physical gels', in contrast with the 'chemical gels' which are completely irreversible systems.

The mechanisms of formation of these physical gels are still not elucidated. There is one feature common to all of them, that is the growth, during the sol–gel transition, of a network of polymer chains linked by physical bonds (hydrogen bonds, Van der Waals forces, etc.) which account for the elastic properties, or the solid-like behaviour of the gels. The macroscopic measurable parameters of the gels are certainly related to their microscopic structure, but the latter depends, not only on the actual thermodynamic conditions (temperature, concentration, pH, ionic force,...), but also on the previous history of the sample. This is one of the major difficulties encountered in characterizing these systems.

The aim of this work is to investigate the structural changes of the gelatin network, during gelation under different thermal conditions. The method used is the optical rotation measurement, at a fixed wavelength. This measure is sensitive to the conformational changes of the polymer during gelation.

We have performed different types of thermal treatments in order to show the influence of thermal history upon the structure of the sample. The polymer concentration, the pH and the ionic force (NaCl concentration) were adjusted to the same values for all samples. The influence of some of these parameters (especially the gelatin concentration) will be considered in the next step of this work.

Polarimetric measurements obtained from collagen solutions for different heating experiments are also reported for comparison with the gelatin gels.

Our results on the kinetics of the structural changes during gelation are examined in terms of the Avrami theory of crystallization[5].

SAMPLE CHARACTERIZATION AND PREPARATION

Our gelatin samples come from lime processed, demineralized ossein kindly provided by Société Rousselot (Aubagne). It is a photographic grade type with the following characteristics:

(i) number molecular weight distribution plotted in *Figure 1*, performed by gel permeation chromatography on Sepharose CL4B column and measured by optical density at $\lambda = 230$ nm; (ii) pH at the isoelectric point 4.9; (iii) humidity: 12%; (iv) ashes: 0.023%; (v) 'Bloom test': 270.

Firstly the samples are swollen for a few hours at 4°C in a solution of water, 0.1 M NaCl (concentration ~5% w/v) and a small amount of sodium azide, NaN_3, added to prevent bacterial contamination. This antibacterial has no effect on gelation. Then the samples are dissolved at 45°C in about $\frac{1}{2}$ h, and the pH adjusted to 7 by adding a

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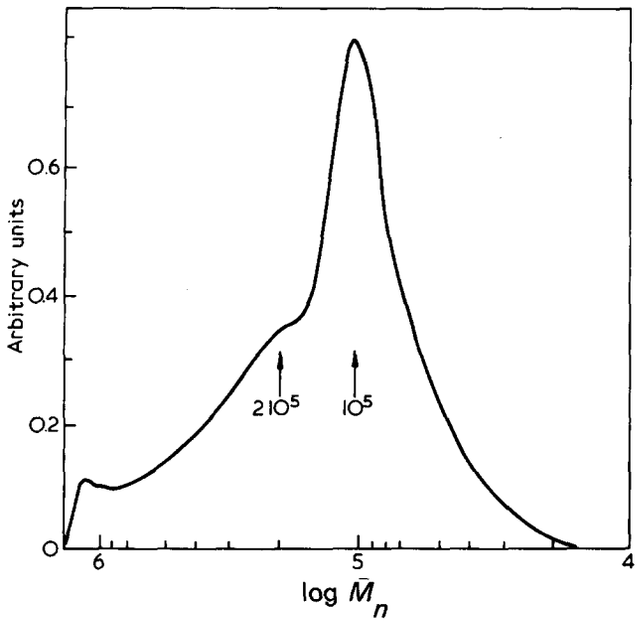

Figure 1 Number molecular weight distribution of the gelatin samples, performed by gel permeation chromatography on Sepharose CL4B column and measured by absorbance at λ = 230 nm (by courtesy of Société Rousselot, Aubagne)

solution of water and NaOH. The final concentration of gelatin lies between 4.5 and 4.7% w/v for all the samples (air-dry protein content).

EXPERIMENTAL METHOD

The optical methods for detecting conformational changes in gelatin or collagen solutions have been widely used in the literature[6]. These conformational changes are the origin of the growth of the infinite network, provided the concentration of the solution is greater than ~1% w/v.

The optical rotation measurement is a simple and powerful method. The optical rotation angle α is related to the helix content of the sample. We have used a Perkin-Elmer 241 MC Polarimeter, working at the wavelength $\lambda = 436$ nm. The optical rotation angle α was automatically recorded with a precision of ±0.001°. The samples of gelatin gels were completely transparent to the eye and allowed accurate measurements, in almost all cases. The cells used had an optical path of 0.1 dm and a volume of 0.1 or 0.5 ml. They were thermostatically controlled by an external bath circulating into the jacketed cells. A thermocouple inserted into the cell gave the temperature of the sample with an accuracy of ±0.1°C.

COLLAGEN STRUCTURE AND MELTING

In order to understand the origin of the conformational changes in the gelatin gels, we first recall the main features of the collagen structure. Gelatin is partially denaturated collagen. The collagen unit is a rod of approximately 2800 Å length made of 3 strands, each having a molecular weight of 100000. Each strand is twisted into a left-handed helix of about 9 Å pitch and all three are wrapped into a super-right-handed helix with a pitch of 86 Å. This particular conformation is stabilized by hydrogen bonds from one chain to another. If this hydrogen bonding is broken, the individual chains collapse. Their conformation, in random coils, is then identical to gelatin in solution at high temperatures ($T > 40$°C). The transition from helix to coil can be induced by heating the collagen solution and easily detected by an important change of the optical rotation, at any wavelength greater than 250 nm.

The specific rotation $[\alpha]_\lambda$ at the wavelength λ is defined by:

$$[\alpha]_\lambda = \frac{\alpha_\lambda}{c.l} \tag{1}$$

where α_λ is the optical rotation angle in degrees, c the concentration in g 100 cm^{-3}, l the optical path in the cell in dm.

In the case of collagen or partially regenerated gelatin this measure is sensitive to the presence of the left-handed helices, the super helix giving little distortion to the chains. In the denaturated solutions, it represents the intrinsic specific rotation of the chains. It is usually admitted that collagen in the native state is 100% helicoidal, with a specific optical rotation $[\alpha]_{589\,nm} = -400 \pm 20°$. For the completely denaturated collagen or gelatin in solution at $T > 40$°C. $[\alpha]_{589\,nm} = -120 \pm 5°$.

Generally, the measurement of $[\alpha]$ at a single wavelength is not sufficient for evaluating the helix content of a protein but, in the case of partially denaturated collagen or gelatin in the course of renaturation, the situation seems more favourable because the rotatory dispersion curves can be represented by a single Drude term. Thus, it has been established that the fraction of chains χ in the helical conformation is given by:

$$\chi = \frac{[\alpha]_\lambda^{\text{observed}} - [\alpha]_\lambda^{\text{denaturated}}}{[\alpha]_\lambda^{100\%\,\text{helix}} - [\alpha]_\lambda^{\text{denaturated}}} \tag{2}$$

independently of the wavelength.

Figure 2 shows the equilibrium melting curve of a calfskin collagen in an acid acetic solution measured at $\lambda = 436$ nm in order to point up the main features of collagen denaturation ($c = 4.5$ g l^{-1}):

(a) the optical rotation α is constant for temperatures below 30°C corresponding to $[\alpha]_{436\,nm} = -800 \pm 10°$ which we admit to be the value for 100% helix;

(b) when the temperature is raised the stability of the structure decreases progressively until the helical conformation is completely lost at $T > 35$°C. The mid-point of the transition is at $T_m = 32$°C.

(c) The melting kinetics of collagen are extremely slow. This feature has already been mentioned by von Hippel and Wong[7] who reveal that 'the collagen→gelatin reversion exhibits by far the slowest rate of helix→coil conversion' ever studied! In our experiment, the time required for reaching an equilibrium value of α in the range $30° < T < 35$°C is up to 30 h, when the temperature is raised by 1°C steps. According to the literature, it is a few minutes for DNA, a few microseconds for α-helices!

(d) Using a different method of temperature variation which is a linear increase at the rate of 0.05°C min^{-1}, we found the mid-point of the transition at about $T_m \simeq 36$°C, which corresponds to an apparent shift of 4°C. This latter value of T_m is the one which is usually adopted for the melting temperature of collagen.

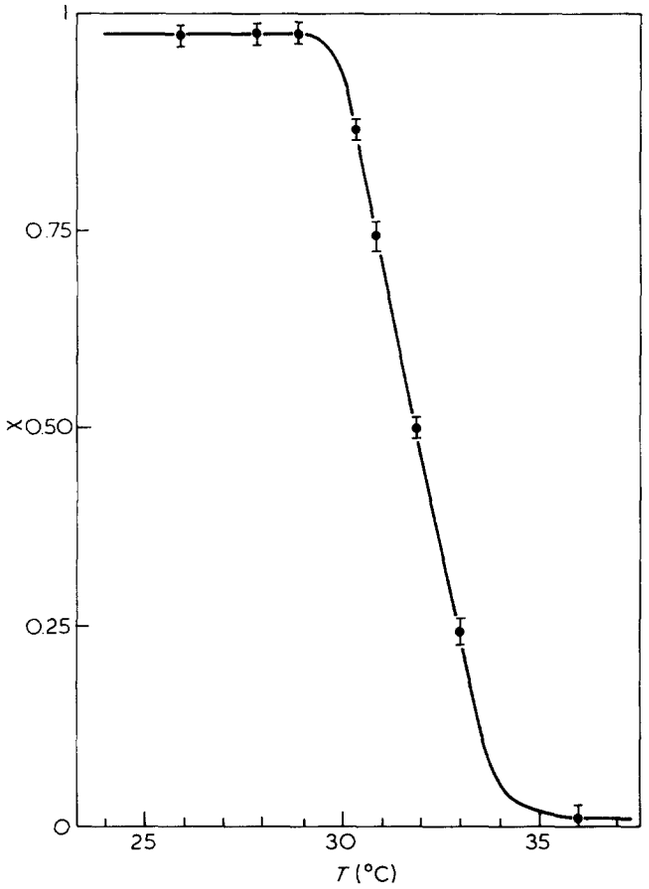

Figure 2 Equilibrium melting curve of calf skin collagen in an acetic acid solution. The helix amount χ is calculated according to equation (2)

GELATIN GELS

Our main work involves the renaturation of gelatin chains in gelling solutions ($c \simeq 5\%$ w/v). A rather detailed study of the renaturation of gelatin gels was presented by Smith in 1919[8] who investigated the kinetics at different temperatures (between 10° and 35°C) and concentrations (1–7% w/v). He concluded the existence of two forms A and B for the gelatin chains (which in fact are the coil and the helix) and reported an 'equilibrium value' of the amount of each form, as a function of temperature and concentration. More recently, Pechlin *et al.* (1963)[9] came to the same conclusion for gelatin gels, while Flory and Weaver (1960)[10] plotted an ultimate value curve of the specific rotation of dilute collagen solutions in water, as a function of the temperature of renaturation.

However, other experimental methods such as rheological measurements[3] or differential scanning calorimetry (d.s.c.)[11] showed no evidence for an equilibrium value of the elasticity or the enthalpy of melting of gels studied under similar conditions.

Another point often mentioned in the literature is the existence of important hysteresis effects for almost all the macroscopic physical parameters, which never regain their initial values, unless the gels are melted to temperatures $T > 40$°C, which completely erase the previous history.

In order to elucidate some of these points, in particular the influence of temperature and time on the gel structure, we have chosen two different methods of gel formation and melting which showed up these effects.

Cooling and heating the gels at constant rates

This method is currently used for the study of thermoreversible gels, associated with optical rotation, fluorescence polarization, n.m.r. etc., for instance for agarose[12–14] or carrageenan gels[15].

The experimental results exhibit an important thermal hysteresis between cooling and heating, the origin of which is not fully understood.

We have investigated the optical rotation changes of gelatin gels as a function of temperature, for different rates of cooling between 0.01 and 0.1°C min^{-1}. The lowest value of v was imposed by the program of temperature control and the highest value was limited by the intertia of the thermostatically controlled bath. The range of temperatures was $1°C < T < 45°C$. *Figure 3* shows two hysteresis curves automatically plotted by simultaneous measurements of the output of the polarimeter and the temperature linearization device for $v = 0.06$°C min^{-1} and $v = 0.1$°C min^{-1}. The corresponding calculated values for χ, the helix content, are indicated on the ordinate axis. These curves nearly coincide for the lowest values of χ, or the highest temperatures, while they depart from each other at the lowest temperatures. The higher values of χ are reached for the lower rate of cooling. The polarimetric measurements become irregular and fluctuating for high degrees of organization ($\chi = 0.6$–0.7) probably due to inhomogeneities in the sample.* This effect can be attenuated by closing the diaphragm of the light source. The width of the hysteresis curve is $\Delta T \simeq 6$°C. Comparison with other gels shows, for instance, in agarose gels, the polysaccharide undertakes a coil double helix transition and the hysteresis on melting is up to 50°C (Arnott et al.[14]). In the low temperature range the polymer is completely in the helical state $\chi = 1$ in contrast with our gels. The ageing process in the agarose gels seems to come from the aggregation side-by-side of the helices, which can induce phase separation phenomena (turbidity, syneresis...). Gelatin gels did not show any opalescence or syneresis even when kept for more than one month.

The method of temperature linear variation seems to indicate in the case of gelatin gels, the balance between the rate of helix growth or melting and the rate of change of temperature. In order to study this behaviour more extensively, one should be able to vary v through a wider range of values. This can be done by using smaller samples when working with high values of v ($v > 0.5$°C min^{-1}) in order to achieve the temperature equilibrium rapidly. A different arrangement for heat transfer would also be needed.

The hysteresis curves are located completely below the limit that can be expected from collagen measurements, 100% helix for temperatures below 30°C, arguing that the gels are non-equilibrium. The next experiments corroborate this statement.

Quenching and annealing the samples

This method has been used in the case of gelatin gels by Godard *et al.*[11] with differential scanning calorimetry (d.s.c.) and by te Nijenhuis[16] with rheological techniques. Our results are presented in *Figures 4* and *5*.

The temperatures investigated are $T = 28$°C, $T = 26.5$°C, $T = 20$°C and $T = 10$°C. The samples were

* The cells examined through crossed polars reveal birefringent domains, located under the tab, which are probably due to local stresses. (This observation was suggested by Dr. C. Rochas who noted the same effects in carrageenan gels.)

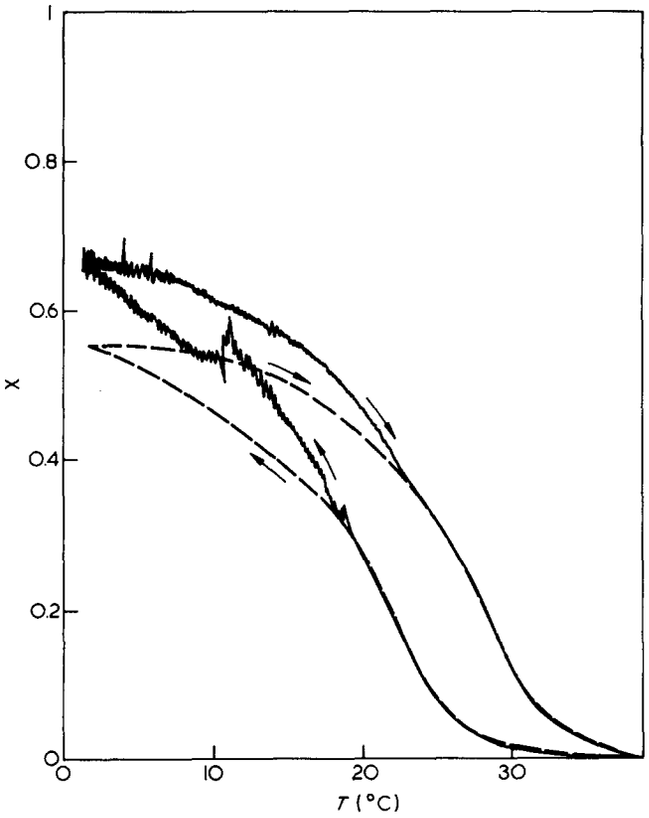

Figure 3 Hysteresis curves obtained by cooling and heating the gels at two different rates: v (—) = 0.06°C min^{-1} and v (---) = 0.1°C min^{-1} (concentration c = 4.6% w/v). The arrows indicate the direction of the temperature variation

first dissolved, then stabilized to $T=40°C$ for a few minutes, finally quenched to the temperatures mentioned and the optical rotation angle continuously recorded. The time required for complete thermal equilibration is of the order to two or three minutes and was checked by the thermocouple inserted within the sample.

The time evolution of the helix content is reported in *Figure 4* for periods of observation of 130 h. The kinetics are strongly temperature dependent. The lowest temperatures provide the most important rates of renaturation, as can be expected from a nucleation-controlled growth.

Although the system is not fully stabilized after this period the rate of evolution decreases. Thus, it has been assumed by many authors, that χ tends towards an asymptotic limit, which is temperature dependent. This idea fails if one plots $[\alpha]$ or χ *versus* the logarithm of the time. No limit appears after long periods of annealing, χ still increases even when the sample is kept during 45 days, as we did for $T=10°C$. Several remarks arise when one inspects these curves:

(a) At the highest temperatures $T=28°C$ or $T=26.5°C$, there is a progressive and slow increase of χ which leads only to the value of $\chi=0.2$ after a period of 100 h.

(b) At lower temperatures, $T<20°C$, very rapid nucleation and growth begin immediately after the quenching, starting while the temperature equilibrium is not already completely achieved. A few minutes after the quenching, the helix content has a rather high value, which represents approximately half of the amount reached during the further 100 h of ageing at the same temperature. This is seen especially in *Figure 5*. The evolution proceeds logarithmically with time for periods between 1 and 100 h (even 1000 h). This has been pointed out by Godard *et al.* who suggested an analogy with the kinetics of bulk crystallization of polymers[17]. We have tried to analyse our results within the same theoretical frame.

THEORETICAL INTERPRETATION

It is possible to analyse the kinetics of a phase transformation in terms of Avrami exponents[5] which fit the data. This type of interpretation is an attempt to explain the structural changes which take place during gelation; it typifies, for example, the existence of crystallization sites in the gel phase.

In our situation, we will describe the kinetics of the phase transformation by a phenomenological equation of the type:

$$\chi=1-e^{-kt^n} \tag{3}$$

where χ is the transformed fraction, k the rate constant and n the Avrami exponent.

The plots of $\log[-\log(1-\chi)]$ *versus* $\log t$ should give a

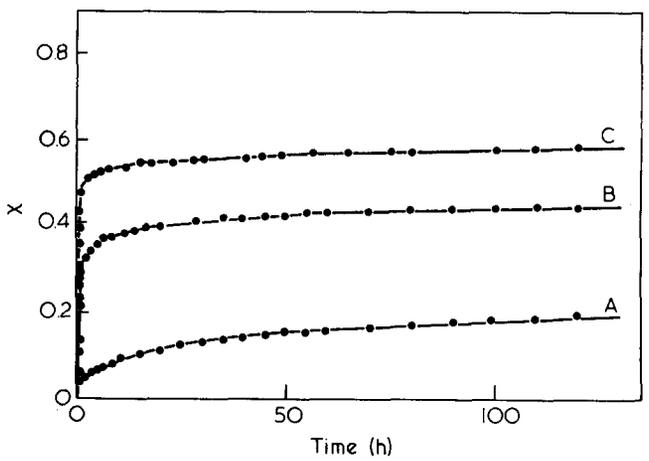

Figure 4 Time evolution of the helix content χ for gels quenched at (A) T = 10°C, (B) T = 20°C and (C) T = 28°C, over a period of 130 h

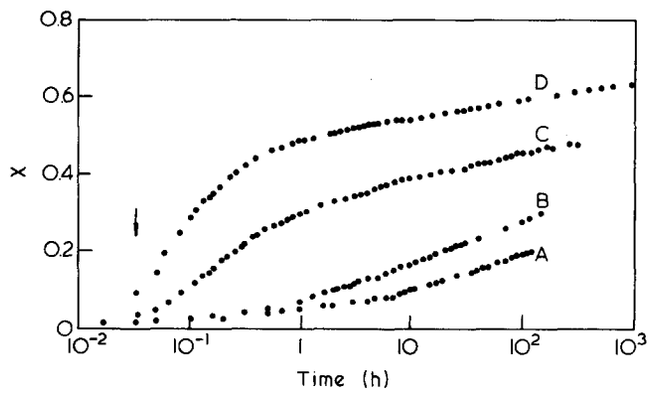

Figure 5 Time evolution of helix content χ (as in *Figure 4*, but the time is in a logarithmic scale) over 10^3 h. (A) T = 28°C, (B) T = 26.5°C, (C) T = 20°C, (D) T = 10°C. The arrow indicates the initial thermal equilibrium

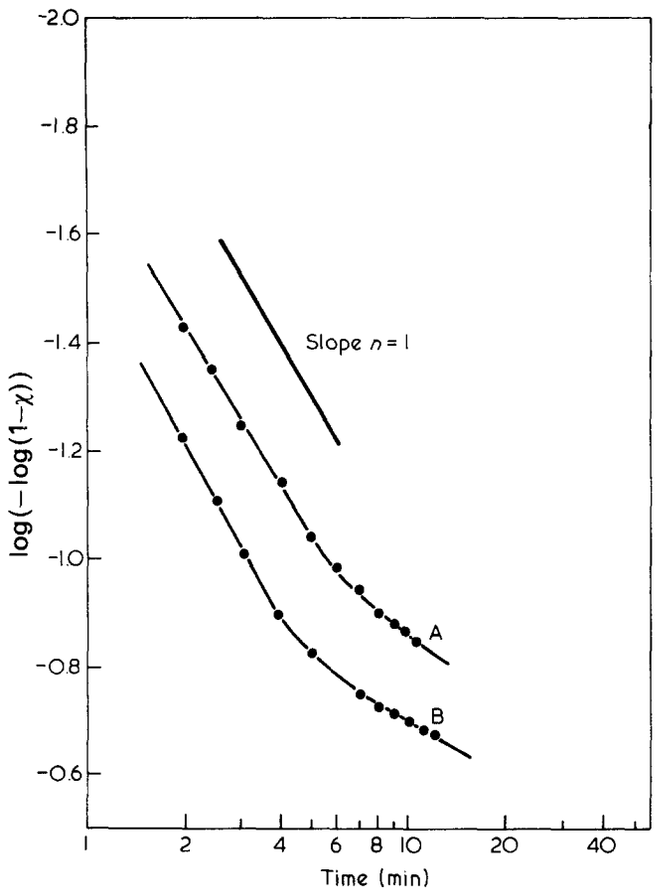

Figure 6 Avrami plot for gels quenched at (A) T = 15.7°C and (B) T = 8°C

straight line of slope n. The value of n depends on the geometry of the crystallites growing (fibres, spherullites, etc.).

The enthalpy of fusion of gels measured as a function of the annealing time, at different temperatures, has been interpreted with this method, and an Avrami exponent $n=1$ was found to fit the first stage of gelation. This was defined as a 'primary crystallization process'[11]. For temperatures below 20°C and a concentration of 5% w/v, this first process has a duration of 5–10 min.

Polarimetric measurements of the helix content χ can be analysed by the same method because the helix renaturation implies the stabilization by hydrogen bonds. Nevertheless, very accurate plots are difficult to achieve for rapid time evolution processes as mentioned above. A minimum of two minutes is required to reach the thermal equilibrium of the sample for cells of 0.1 ml volume. The d.s.c. technique using samples of 10–20 mg allows thermal equilibration within a few seconds which is more favourable. *Figure 6* shows two Avrami plots from measurements at $T=15.7$°C and $T=8$°C, and one can find a slope of approximately $n=1$ during the first minutes of the phase transformation, in good agreement with the d.s.c. results (but with a poorer precision). The same analysis was applied to the higher temperatures $T=26.5$°C and $T=28$°C. In this case, the polarimetric measurements seem much more favourable as the kinetics are slow compared to the thermal equilibration time and only a small amount of helix is renaturated. We expected that the fit would agree for longer periods, however, this was not the case, as seen in *Figure 7*. It appears that no Avrami exponent can be defined to fit the transformation for a reasonable period of time. The structural changes at high temperatures have a much slower evolution, thus suggesting that the stability of the renaturated helices should be improved. At low undercooling, the number of nucleated (left handed) helices along a single chain is reduced and the growth relatively slow. This situation enhances the growth structures having the stability of the collagen fold, the three chain super helices. The melting curves in *Figure 8* support these ideas. We annealed two samples for a period of ~100 h, one at $T=22$°C (gel A), the other at $T=26.5$°C (gel B). Then the gels were melted (at a rate of 0.05°C min^{-1}) and the melting curve recorded by optical rotation measurements. *Figure 8* shows the differential of these melting curves for gels A and B and for a collagen solution (at the same heating rate). It arises from these curves, that two components are present in these gels with different thermal stabilities:

(a) One component with a broad melting curve centred at $T_m=29$°C for gel A and $T_m=32$°C for the gel B which we attribute to the chains aggregated by disordered hydrogen bonds. The broadness of the peak indicates a large distribution of the sizes and degrees of stabilization of the aggregates. The melting point position depends on the quenching temperature, the gel quenched at the lower temperature being the less stable. (b) A second and distinct component, with a melting point centred around $T_m=36$°C, appears for both gels. This component is well detached in the case of gel A and partly superimposed on the broad line in the case of gel B. Its position, compared to the melting curve of collagen, suggests that this component has the structure and the stability of the collagen triple helix fold. This analysis agrees with the results obtained by Beier and Engel[18] on the renaturation of collagen solutions investigated by optical rotation, sedimentation and pepsin attack.

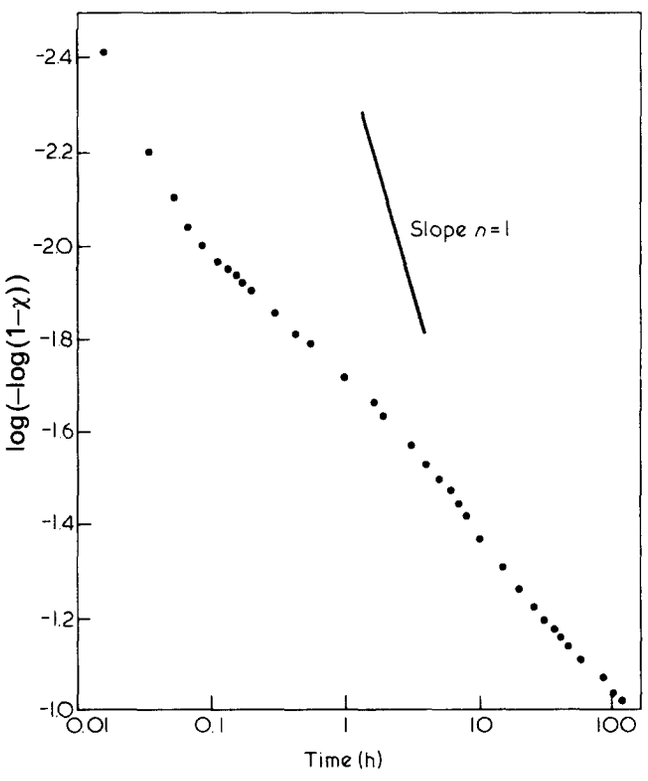

Figure 7 Avrami plot of a gel quenched at T = 28°C

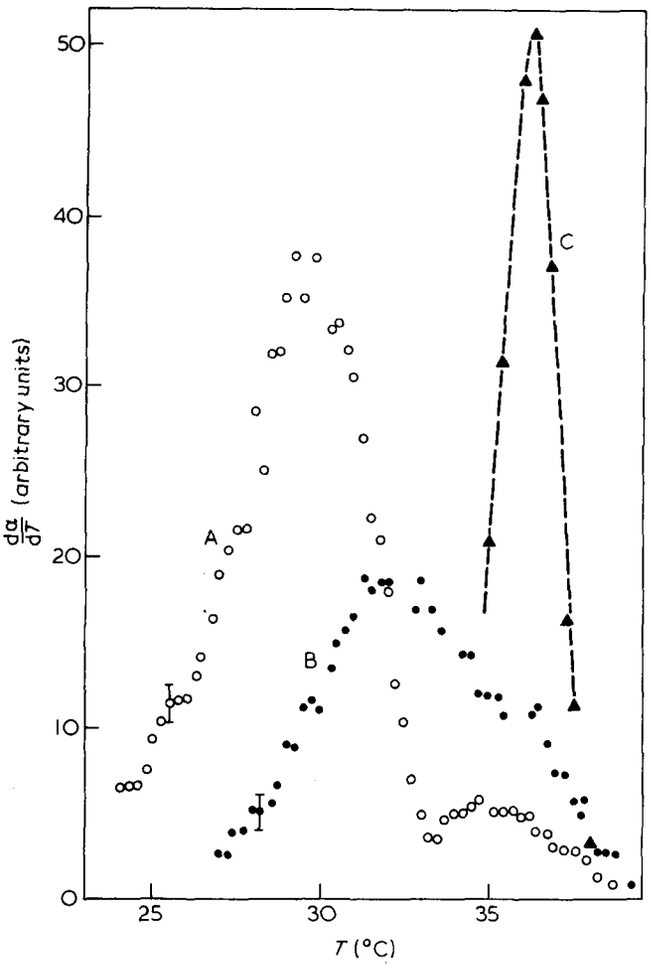

Figure 8 Differential melting curves for two gels annealed during 100 h at (A) $T = 22°C$, (B) $T = 26.5°C$ and (C) for a calf skin collagen solution; heating rate $v = 0.05°C\ min^{-1}$

CONCLUSION

The purpose of this paper was twofold. Firstly we wished to make a statement on the main results known on collagen or gelatin solutions and gels, by optical rotation measurements, as a great deal of experimental work has been devoted to this subject in the past. Secondly we wanted to compare our measurements to results obtained more recently by different methods (d.s.c., rheological methods,...) in order to find a correlation between them, and elucidate some contradictory aspects.

Our experimental work was especially directed towards the investigation of structural changes of gels, as a function of the thermal treatments. Progressive cooling at constant rates or quenching and annealing at constant temperatures were followed by optical rotation measurements and the helix amount calculated, by comparison with collagen.

The different thermal treatments which have been applied proved that the gels were still in non-equilibrium even after periods of anneling of hundreds of hours (up to 1000).

The amount of helix renaturated χ is strongly temperature dependent, the higher amounts for the lower temperatures being $\chi \simeq 0.7$ at maximum in our experiments. The helices are stabilized by hydrogen bonds which can be either intra- or inter-molecular bonds. To our 5% w/v concentration, the bonds are mainly inter-molecular, involving the aggregation of the chains and the formation of the gels. The kinetics of formation of the gels and their melting curves reveal two different mechanisms of helix stabilization:

(a) At low temperatures, $T < 20°C$, a rapid association of chains by disordered hydrogen bonds giving gels with low temperature melting points (generally $< 30°C$). The first stage of the transformation can be interpreted by an Avrami exponent $n = 1$, by analogy with the crystallization theories.

(b) At high temperatures, $T > 20°C$, the renaturation of the helices is much slower and partially allows the growth of structures having the thermal stability of collagen.

Further work involving wider ranges of temperature and concentration is in progress (polarimetric and rheological measurements).

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