Study of junction zones in gelatin gels through selective enzymatic digestion

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A full topological description of gelatin gels requires knowledge of the number, size and functionality of the helical segments, which act as effective junction zones. Selective enzymatic attack on renatured gelatin has allowed us to isolate the helical segments by size exclusion chromatography. For sharp fractions, the size of the helical segments is almost independent of initial molecular weight and is about 100–200 amino acid residues for each strand. This size does not depend very much on concentration, but increases slowly with renaturation time and with temperature. We believe that helical segments are always formed by the same process: in accordance with previous kinetic studies, the starting point would be a bimolecular nucleation. For such nucleation, two strands are necessarily antiparallel, and this can explain the limited length of the helices.

(Keywords: gelatin; enzymatic digestion; triple helix size; renaturation; helical structure; enzyme activity)

INTRODUCTION

Among the variety of natural polymers capable of forming physical gels, gelatin is a very interesting example that has been extensively studied[1,2].

During renaturation of gelatin solutions at low temperature, 'imperfect triple helices' are formed, similar, at first sight, to the parent collagen triple helix. When the concentration is high enough, these triple helix segments act as junction zones, the connectivity of the system increases and eventually there is gel formation. A precise description of these gels requires knowledge of the number and the functionality of the junction zones.

The simplest idea is that each triple helix connects three different chains, but recent kinetic studies by optical rotation[3] suggest that the process is bimolecular rather than trimolecular. Classical optical rotation measurements give the fraction of amino acid residues within helical segments[4], but the determination of the number of such segments requires knowledge of their length. Consequently, we have tried to obtain direct information on helical segment size by selective enzymatic degradation of renaturated gelatin samples.

It is well known that rigid structures are much more resistant than disordered ones, since enzymes need proper spatial interaction with the substrate to be active. Engel and coworkers[5] have studied the coil–helix transition of acid-soluble calf skin collagen. On pepsin attack, the helical parts were resistant while disordered chains were degraded. More recently, similar behaviour has been observed using trypsin[6].

By this method, the sample can be separated into two fractions: a highly degraded part of very low molecular weight and a resistant portion of higher molecular weight. As helix formation is a thermoreversible process, careful temperature control is required during renaturation, enzymatic digestion and s.e.c. analysis.

MATERIALS AND METHODS

Several gelatins from limed bone ossein were kindly supplied by Rousselot: R1, R2 and R3 are respectively first, second and third extracts. Gelatin M, supplied by Merck, was a low-molecular-weight sample from bovine hide.

Pepsin A (EC 3.4.23.1) from porcine stomach mucosa and trypsin (EC 3.4.21.4) from bovine pancreas type III (dialysed and lyophilized, essentially salt-free) were obtained from Sigma.

Sample preparation

The solutions were prepared by weighing gelatin in a volumetric flask and adding the water needed. After swelling for 4 h at ambient temperature, gelatin was dissolved under stirring at 50°C for about 10 min. An average value of 12% w/w moisture content was taken into account.

Size exclusion chromatography

The analytical apparatus used as eluant H_2O/0.1 M $NaNO_3$/sodium azide (200 mg l^{-1}) at a flow rate of 1 ml min^{-1}, with an RI detector (Waters R410). The injection loop (150 μl) (Rheodyne) and column set (one 30 cm column TSK PW5000 + Waters UHG250) were carefully thermostated in a water bath to ± 0.1°C. Pullulan standards were used for calibration. This calibration curve gives correct molecular weights for soluble collagen α chains and for fragments from CNBr cleavage, and has therefore been used without further modification.

A preparative column (1 m × 20 mm i.d.) packed with Fractogel HW55S was used to obtain sharp fractions, using the same eluant as previously at a temperature of 45°C and a flow rate of 1 ml min^{-1}. We made repetitive

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injections of a 5 mg ml^{-1} R1 gelatin solution (20 ml each), and 20 different 10 ml fractions were collected.

Optical rotation

Optical rotation (α) was measured on a Jobin-Yvon RJ digital polarimeter at wavelength $\lambda = 589$ nm, using jacketed cells of pathlength 0.20 or 0.70 dm. Temperature was controlled by a circulating water bath and measured by a thermocouple situated directly in the cell.

PRELIMINARY STUDIES ON DENATURED GELATINS

First it was necessary to define the experimental conditions needed to ensure sufficient degradation of the disordered part of gelatin by enzymatic attack.

Temperature

This parameter plays a trivial role in the temperature range explored. For most enzymes, near ambient temperature a jump of 10°C doubles the digestion rate. Consequently, results obtained at 37°C on denatured samples can be extrapolated to any temperature. Enzymatic attack is slower but still efficient even at very low renaturation temperature (5–10°C).

pH

Optimization of this parameter is crucial, as each enzyme exhibits an optimum activity at a given pH and is totally inactive far from this optimum value.

The optimum activity for trypsin is near pH 7, which is close to that of the gelatin solutions (pH 6), and consequently no special adjustment was needed.

For pepsin, the optimum activity is near pH 1.5 and this enzyme is denatured above pH 5. Very acid pH is unsuitable, owing to rapid hydrolysis of gelatin, leading obviously to non-selective degradation. In addition, renaturation can also be affected by large pH changes. After a series of tests, pH 4 was selected as the best compromise. In gelatin solutions at this pH, no significant degradation occurs even after one week at 20°C and pepsin activity, although slow, is still efficient.

Enzyme/gelatin ratio

To obtain very efficient degradation of the disordered part, a large enzyme/gelatin ratio is preferable. The only limitation is interference between the enzyme peak and the gelatin peak in chromatograms. For this reason, we have generally used a ratio of 1/10 w/w.

Digestion time and maximum extent of degradation

Pepsin attack on denatured samples at 30°C leads to slow degradation. A stable situation is observed after 100 h and s.e.c. molecular weights of the degraded sample are $M_n = 5000$ and $M_w = 10\,000$. This is consistent with the specific cleavage of peptide linkages at phenylalanine and tyrosine residues[7]. For most gelatins, these two aromatic amino acids represent 2.5% of the total, giving an expected DP_n of 40 ($M_n = 4000$).

Trypsin is able to cleave a wide variety of peptide bonds, and its attack is much more rapid: a stable situation is observed after 30 min at 30°C, with $M_n = 1100$ and $M_w = 2500$ for the resulting degraded sample.

Arresting digestion

It would be useful to stop the digestion process at any time to observe the different stages of degradation. Classical chemical inhibitors (iodoacetamide, diisopropylfluorophosphate) have been tested[8] by adding successively to an enzyme solution the inhibitor and, immediately after, a denatured gelatin. It has never been possible to stop the enzyme activity totally and we have observed only a decreased rate of gelatin degradation. Other methods such as temperature jumps or pH jumps are not attractive as they would change the triple helix content. Ultimately, the only way was to use sufficiently rapid analytical methods.

RESULTS ON RENATURED SAMPLES

For a typical experiment, sample aliquots were characterized by s.e.c. analysis and optical rotation at a given time after isothermal renaturation. Enzymatic digestion was then performed, and the resulting mixture analysed by s.e.c.

A first series of experiments was carried out using a set of sharp fractions from R1 gelatin of molecular weight between 40 000 and 300 000. Two temperatures were tested: 15 and 20°C. The concentration of the gelatin solution was 2 mg ml^{-1}, well below gelation threshold, to allow rapid mixing with the enzyme.

Pepsin digestion

As pepsin attack is very slow, this enzyme was used only after a long renaturation time (four weeks). Sample pH was adjusted to pH 4 with dilute acid just before adding pepsin at an enzyme/gelatin ratio of 1/10.

As shown in *Figure 1*, just prior to attack, large amounts of high-molecular-weight aggregates are formed. After sufficient attack, we observed a bimodal chromatogram corresponding to a degraded fraction and a resistant fraction of higher molecular weight (*Figure 2*). After one week, the peak area and position of the resistant fraction are stable, and no additional change is observed for times as long as one month.

We believe that the resistant fraction contains large amounts of triple helix segments and that the gelatin calibration curve cannot therefore give real molecular weights. For that reason, we indicate only apparent M_n and M_w. The observed results are summarized in *Tables 1* and *2*.

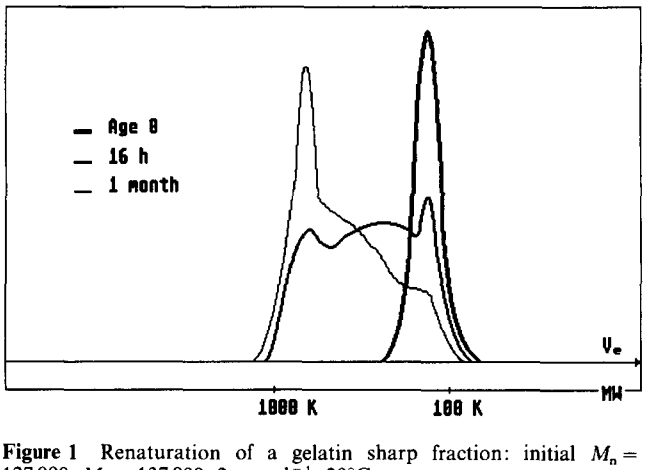

Figure 1 Renaturation of a gelatin sharp fraction: initial M_n = 127 000, M_w = 137 000, 2 mg ml^{-1}, 20°C

After digestion, we observed that the weight fraction of the resistant part was very close to the helical content, indicating that the resistant part is mostly formed of helical segments, as expected. Molecular size in the resistant part is higher at 20°C than at 15°C. More surprisingly, gelatin fractions having different molecular weights give resistant parts with very similar apparent values of M_n and M_w. This indicates clearly that the length of each helical segment is smaller than that of the fraction of lowest molecular weight (38 000). To estimate the true molecular weight of each triple helix strand, we quickly analysed the digested mixture immediately after brief heating (4 min at 50°C). In this way, we allowed denaturation of the resistant part with only very limited attack by the pepsin still present. The result is shown in *Figure 3*. Unfortunately, there is insufficient separation between the denatured segments and the degraded part. We observe just a shoulder at a molecular weight around 10 000 to 20 000.

Trypsin digestion

Digestions on 2 mg ml^{-1} samples renatured for one month at pH 6 were performed at 20°C using a trypsin/gelatin ratio of 1/10. Digestion is now much more rapid. The run time for s.e.c. analysis is 20 min, but we can consider that the reaction is stopped only a few minutes after injection. Consequently, the estimated digestion time is just the time between trypsin addition and s.e.c. injection. *Figure 4* shows that a resistant part exists; its peak position is stable after 25 min but its area decreases continuously. Values of apparent M_n and M_w are still independent of the starting molecular weight of

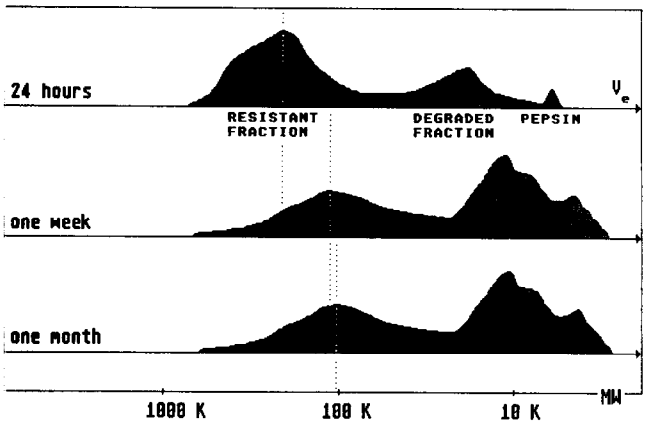

Figure 2 S.e.c. analysis of pepsin digestion products at different times: fraction, temperature and concentration as in *Figure 1*; renaturation time, one month; digestion time, as indicated

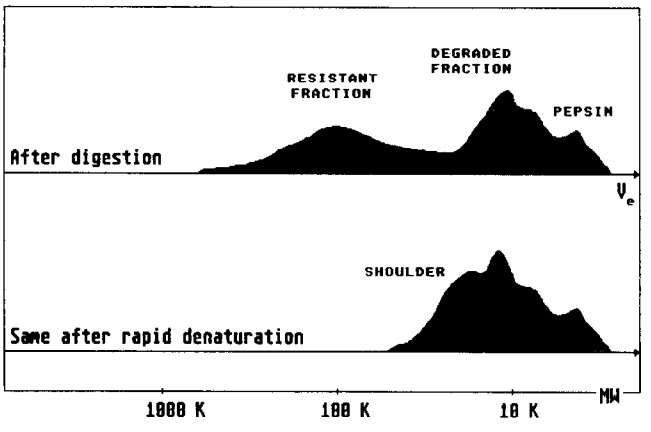

Figure 3 Effect of denaturation on resistant fraction: same fraction and concentration as in *Figure 1*; 15°C; renaturation time, one month; digestion time, one week

Table 1 Results of pepsin digestion: 15°C; renaturation time, one month; pepsin digestion time, one week; pH 4

Fraction		Polarimetry:	Resistant fractions		
$\bar{M}_n$ ($\times 10^3$)	$\bar{M}_w$ ($\times 10^3$)	Helix content	Relative peak area	S.e.c. $\bar{M}_n$ ($\times 10^3$)	S.e.c. $\bar{M}_w$ ($\times 10^3$)
266	309	0.51	0.60	114	178
182	213	0.51	0.58	111	173
127	137	0.53	0.56	110	179
99	104	0.57	0.56	105	162
81	83	0.55	0.54	104	159
65	68	0.48	0.53	98	144
38	39	0.44	0.51	84	121

Table 2 Results of pepsin digestion: 20°C; renaturation time, one month; pepsin digestion time, 3 days; pH 4

Fraction		Polarimetry:	Resistant fractions		
$\bar{M}_n$ ($\times 10^3$)	$\bar{M}_w$ ($\times 10^3$)	Helix content	Relative peak area	S.e.c. $\bar{M}_n$ ($\times 10^3$)	S.e.c. $\bar{M}_w$ ($\times 10^3$)
266	309	0.52	0.45	160	245
182	213	0.48	0.43	154	226
127	137	0.43	0.42	150	237
99	104	0.48	0.41	175	269
81	83	0.46	0.38	155	219
65	68	0.46	0.35	144	200
38	39	0.40	0.39	134	178

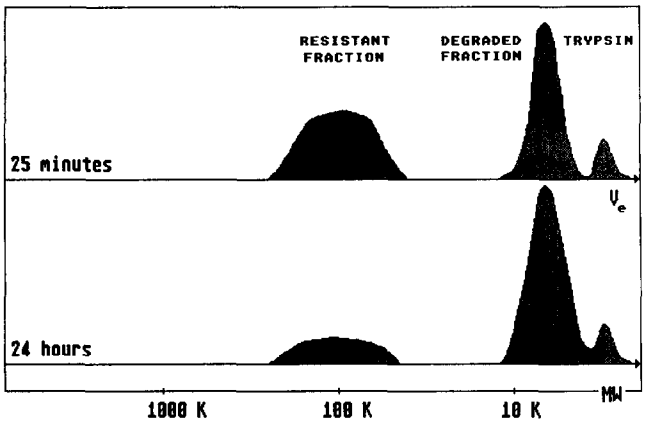

Figure 4 S.e.c. analysis of trypsin digestion products at different times: same fraction, temperature and concentration as in *Figure 1*; renaturation time, one month; digestion time as indicated

Table 3 Results of trypsin digestion: 20°C; renaturation time, one month; trypsin digestion time, 25 min; pH 6

Fraction		Resistant fractions	
$\bar{M}_n$ ($\times 10^3$)	$\bar{M}^w$ ($\times 10^3$)	S.e.c. $\bar{M}_n$ ($\times 10^3$)	S.e.c. $\bar{M}_w$ ($\times 10^3$)
266	309	100	142
182	213	104	146
127	137	102	154
99	104	96	136
81	83	98	140
65	68	96	146

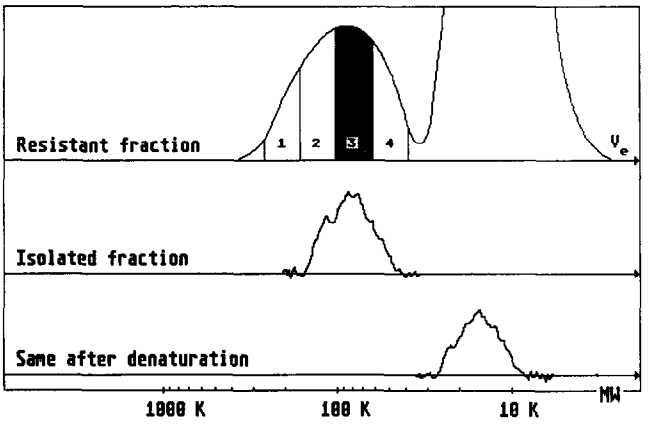

Figure 5 Effect of denaturation on an isolated fraction of resistant part: gelatin R1; 5 mg ml^{-1}, 20°C; renaturation time, one week; digestion time, 20 min; s.e.c. fractionation using the analytical apparatus; volume of each fraction, 0.8 cm^3

the fraction studied (*Table 3*); for this reason further studies were carried out using unfractionated gelatins.

To obtain more precise information on the resistant part, it was necessary to isolate it. For this purpose, a 5 mg ml^{-1} R1 gelatin solution was digested by trypsin and immediately fractionated by s.e.c. The fractions corresponding to the resistant part were then analysed, either without any temperature change or after denaturation. Results presented in *Figure 5* and *Table 4* confirm the conclusions from the preliminary study using pepsin. The molecular weights of separated strands are centred around 20000. In addition, the increase in the ratio of the apparent peak M_p values before and after denaturation clearly confirms the rigid structure of the native fractions.

By summing all the denatured fractions we obtain $M_w = 20000$ and $M_n = 12000$. Unfortunately, we have no direct evidence to decide if, after denaturation, triple helices give three equal coils, or two coils, one of which is twice as large as the other as expected for a bimolecular structure[3]. Even in the second situation, two peaks would not be observed, owing to the polymolecularity of the collected fractions and to the imperfect resolution of the s.e.c. apparatus. In addition, the signal level is too low to allow us to obtain very precise polymolecularity index values.

The influence of renaturation time was also studied on R1 gelatin at a concentration of 20 mg ml^{-1}. Just prior to trypsin attack the gel was diluted to obtain a final concentration of 2 mg ml^{-1}. *Figure 6* and *Table 5* show the observed apparent M_n and M_w for the resistant part. There is a moderate increase in M_w with renaturation time. Correspondence between helix content and relative amount of resistant material is still observed.

Similarly we have checked the influence of concentration over the range 2 to 100 mg ml^{-1} for 24 h

Table 4 Trypsin digestion and s.e.c.: fractions defined as in *Figure 5*

Fraction ref.	1	2	3	4
M_{peak} native ($\times 10^3$)	200	120	80	50
M_{peak} denat. ($\times 10^3$)	26	20	17	12
Ratio	7.7	6.0	4.7	4.2

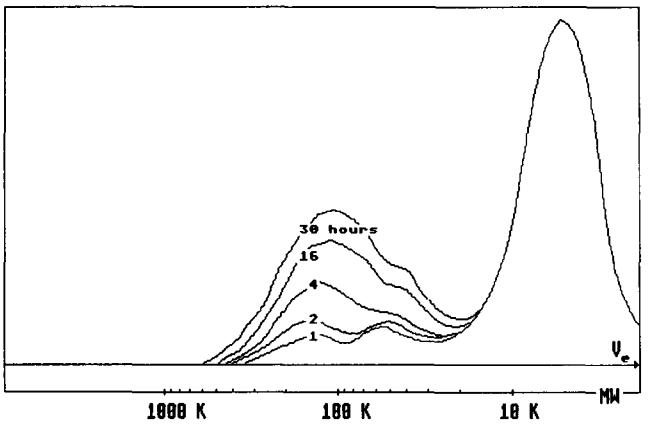

Figure 6 Resistant fraction surface *versus* renaturation time: gelatin R1; 20 mg ml^{-1}; renaturation times as indicated; 20°C; trypsin digestion time, 25 min

Table 5 Trypsin digestion: resistant fraction *versus* renaturation time (*Figure 6*)

Renaturation time (h)	$\bar{M}_n$ ($\times 10^3$)	$\bar{M}_w$ ($\times 10^3$)	Relative peak area	Helix cont.
1	71	109	15	12
2	79	128	21	18
4	91	144	24	23
16	98	165	29	31
30	176	243	38	36

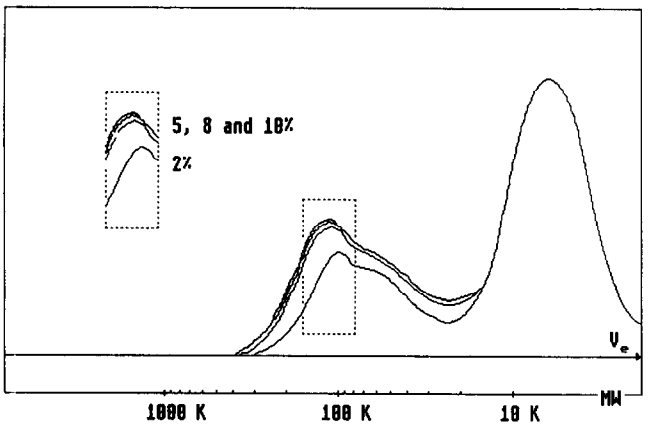

Figure 7 Resistant fraction *versus* gelatin concentration: gelatin R1; concentration as indicated; renaturation time, 24 h at 20°C; trypsin digestion time, 1 h

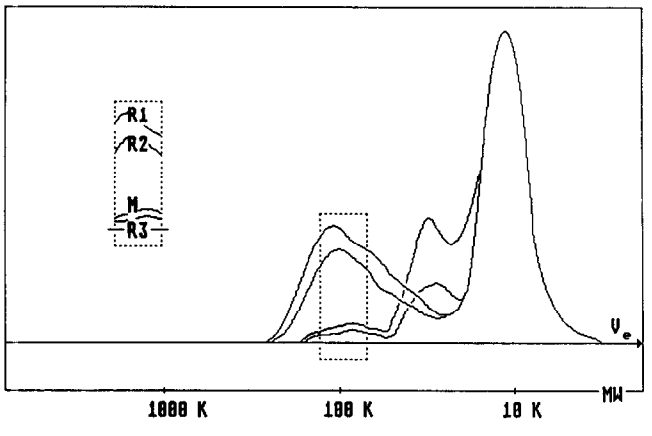

Figure 8 Resistant fraction of different gelatins: Rousselot gelatins R1, R2 and R3; Merck gelatin M; concentration, 20 mg ml^{-1}; renaturation time, 24 h at 20°C; trypsin digestion time, 1 h

renaturation at 20°C. Longer digestion times were used to allow attack on strong gels, but after 1 h we observed apparent values of $M_n = 80\,000$–$100\,000$ and $M_w = 150\,000$–$200\,000$ for the resistant part whatever the concentration (*Figure 7*).

Finally, we have observed that other gelatins give roughly the same results. Some of them renature very slowly and give low amounts of resistant product; the apparent molecular weights, however, are always limited. The intermediate peak observed (*Figure 8*) for the highly degraded gelatins M and R3 will require further investigations.

CONCLUSIONS

After enzymatic digestion, renatured gelatin samples exhibit a resistant part, which consists mainly of helical segments. This study clearly shows that the length of these helical segments is severely limited, whatever the molecular weight of the original gelatin, and lies around 100–200 amino acid residues for each strand for temperatures around 20°C. A first explanation for this limited length could be the time for *cis–trans* isomerization of prolines[9], which allows multiple nucleations to occur along a given molecule and prevents helices from reaching large sizes.

But one additional explanation is related to the

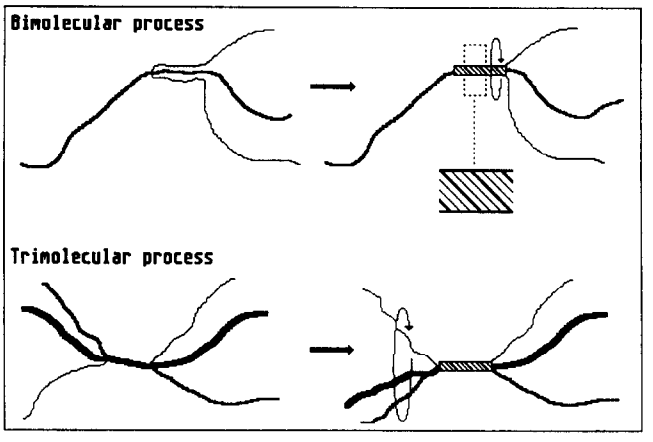

Figure 9 Comparison between bi- and trimolecular processes in helix formation

proposed bimolecular nucleation process. In this case, two of the three strands have to be antiparallel, and consequently defects preventing the growth of the helix can be accumulated. Unfortunately, experimental limitations have not allowed us to observe directly that each helical segment gives, after denaturation, two coil populations with molecular weights in the ratio 2/1.

It is striking that the size of the helices does not depend strongly on concentration. This indicates that the main process is always the same, and is an additional argument for bimolecular nucleation. Indeed, a trimolecular process would be very highly improbable in dilute systems.

The size of the helical segments increases slightly with temperature, consistent with the fact that the minimum chain length required to give a stable helix increases with temperature[10,11]. This observation is in agreement with independent studies showing an increase in the melting point with increasing renaturation temperature[4,12].

Helical segment length also increases with renaturation time, consistent with the possibility of unwinding of small helices, which can then be slowly replaced by larger, more stable ones[3]. This idea is confirmed by the progressive decrease in the resistant fraction during trypsin digestion.

We therefore propose as a general scheme for gelatin gel formation a bimolecular nucleation process starting from a kink on one coil. Taking as fixed reference the three disordered strands on the growing end, helix growth can occur by simple rotation of the existing helical segment (*Figure 9*). By contrast, a trimolecular structure, with three disordered chains at both helix ends, would need large cooperative motion at one end of the helix.

More generally, with a bimolecuar nucleation process there is no limitation to increasing connectivity of the system, as there is no spatial constraint on the disordered parts. For a trimolecular process, growth of several helices joined together by the same disordered segment would impose a number of topological conditions very difficult to fulfil.

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