Studies on the Conformational Transitions of Rat Skin Collagen using a Spin-label Probe

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A technique is reported for spin labelling salt-soluble rat-skin collagen and using e.s.r. spectroscopy to study its conformational properties. The covalently labelled collagen undergoes a series of temperature-induced transitions which parallel those observed for native material. Three discrete processes are evident which are in accord with current view on the molecular unwinding of the tropocollagen triple helix into random coils. The temperature dependence of the e.s.r. spectra yields activation energies related to the expansion of the micro-environment around the probe. Evidence for the reversible destabilisation of spin-labelled tropocollagen by acetic acid, urea, and guanidine hydrochloride is presented.

COLLAGEN is the major structural protein of connective tissue and bone. It has become apparent from recent studies that there are at least four types of collagen and thus it is not a unique molecule but rather a family of related molecules. The triple stranded helix forms the common structural unit at the molecular level for all types. Each of the three α-chains consists of *ca.* 1 000 amino-acid residues with the stability of the helical structure a consequence of the regular Gly–X–Y triplet array in the amino-acid sequence. Type I collagen, isolated from animal or human skin, tendon, and bone,[1] is composed of two identical α1 (I) chains and one α2 chain. A second type of collagen extracted from a wide variety of cartilages [2-5] consists of three identical α1 (II) chains which bear a close structural and biological relationship to the α1 (I) chains. Human dermis,[6] aortas,[7] and insoluble skin [8] contains a third type of molecule built on three α1 (III) chains. Collagen of a fourth type has been found in basement membranes.[9] The biological roles of the various forms of collagen in normal and disease states have been extensively reviewed.[10,11]

The various types of collagen molecules, in common with many other proteins, exhibit heat lability in solution. This results in intramolecular transitions to less organised structural forms at characteristic transition temperatures such as the gel point. Thermally induced conversions of rigid triple helix collagen molecules to random coil forms (tropocollagen→gelatin transition) have been studied by a variety of physical techniques including enzymatic digestion,[12] measurement of physical parameters-solution viscosity,[13] polarimetry,[14] and sedimentation velocity [15] or, alternatively, spectroscopic methods including o.r.d. and c.d.,[16,17] laser scattering,[18] and proton and carbon-13 n.m.r.[16,19,20] Collectively all these techniques have provided a uniform picture of the molecular dynamics of the random coil and helical states transition with ^{13}C n.m.r. in particular, providing detailed information at the residue level. In neutral salt solution on heating to *ca.* 30 °C, collagen forms reversibly an elastic gel with properties very different from gels formed on cooling denatured collagen. At *ca.* 40 °C the soluble collagen chains unravel to form a solution of parent gelatin. Further heating to *ca.* 65 °C results in soluble gelatin in which the chains become random disorientated strands. For compendia of recent physicochemical studies describing these transitions see refs. 21 and 22.

While these techniques can detect and monitor conformational changes they give little quantitative information on the extent of such changes. By contrast, spin-label probes can be used to provide this type of information.[23,24] The e.s.r. spectrum of a spin-label probe is very sensitive to its mobility and thus when attached to a protein can provide direct evidence for conformational changes.[25] It was thus anticipated that

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the conformational transitions of collagen could be further elucidated using a spin-label probe. An additional advantage of spin-label probes is the inherent sensitivity of e.s.r. methods where concentrations of *ca.* 10 μM label provide easily accumulated signals (*cf.* 10 mM for n.m.r. studies). Furthermore, it is well recognised that both the hyperfine interaction tensor and the *g*-factor tensor of the nitroxide radical in a polymer matrix are anisotropic. Both are averaged dependent on the rate of rotational motion of the nitroxide radical within its local environment and result in a variation of the e.s.r. spectrum with temperature.

Very recently Nagamura and Woodward have reported [26] studies on the motion of cattle hide collagen films over a wide range of conditions of temperature and water content using the spin-probe, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl. This probe, when embedded in a polymer matrix permits the molecule motions of the matrix to be monitored in terms of the line shape response and has been successfully used in a variety of applications with synthetic polymers. In contrast to the spin-labelling method, the spin probe molecules are not chemically bound to the matrix but held by physical forces.

In the present study, we have investigated the properties of a covalently modified and purified salt-soluble rat-skin collagen $\{\alpha 1(I)\}_2\alpha 2$ using *N*-(1-oxyl-2,2,6,6 tetramethyl-4-piperidinyl)iodoacetamide (I) as a spin label. We were interested in following the thermal transitions between triple stranded helical and random coil structures. We also sought to determine the effect of various chaotropic reagents on the labelled material. Finally, the question of enzymatic fragmentation was briefly examined. A preliminary account of some of the findings of this study has been reported.[27]

Materials and Methods.—The collagen chosen for this investigation was obtained from adult male rats. Neutral, salt-soluble rat-skin collagen was prepared following the method of Henneman and Nichols.[28] The final product, reprecipitated twice from 20% NaCl, was dialysed against deionised water and freeze dried. A comparison of the properties of this material to other native collagens has been described elsewhere.[29]

Spin-labelled samples for the e.s.r. study were prepared in batches and stored at −20 °C. The purified rat-skin collagen (10 mg) was dissolved in 10% NaCl and 0.05M-KH_2PO_4 buffer (pH 7, 2 ml) at 0 °C and the spin label (I) (0.2 mg) added. The solution was heated at 25 °C for 3 h. As neutral salt-soluble rat-skin collagen in 10% NaCl gels slowly at 25 °C, the solution was periodically removed from the incubation bath, cooled to 10 °C and stirred before re-warming to 25 °C. The reaction mixture was then exhaustively dialysed successively against the 10% NaCl–0.5M-KH_2PO_4 buffer, 10% NaCl–0.05M-Tris (pH 7.2), and 10% NaCl (2 × 1 l each) to remove excess or unbound label; the solution was then centrifuged at 20 000 *g* for 30 min. The hydroxy-proline content (w/w%) of the spin-labelled collagen was 12.7 compared to the reported value [30] of 12.1 for salt-soluble rat-skin collagen. Spectra of the labelled salt-soluble rat-skin collagen were recorded at a concentration of *ca.* 2 mg/ml. In analogous preparations, fibrous spin-labelled collagen was precipitated by adding NaCl to the dialysed and centrifuged solution to a final concentration of 20% NaCl and the reprecipitated product collected as above. Fibrous spin-labelled collagen was hydrated in a 25% water–ethanol mixture at a concentration of *ca.* 2 mg ml^{-1}. Similarly, a 0.5M-acetic acid solution was prepared from a lyophilised sample following an additional dialysis of a portion of the reaction mixture against distilled water.

Bacterial collagenase (Worthington) and trypsin (Sigma Chem. Co) were commercial samples. Reagents used were all analytical grade. The abbreviations, Tris and GdHCl refer to 2-amino-2-(hydroxymethyl)-1,3-propanediol and guanidine hydrochloride respectively. *N*-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide and 1-oxyl-2,2,6,6-tetramethyl-4-aminopiperidine were purchased from Syvar, Palo Alto, California.

E.s.r. measurements were made using a 500 μl aqueous cell and a Varian E-4 X-band spectrometer. The temperature was regulated from 0—49 °C (±0.5 °C) with a Varian E-257 variable-temperature accessory. Correlation times (τ_c) were calculated using the method of Stone *et al.*[25] The stated errors are the standard deviations from the line of best fit through all data points utilised in the least-squares analysis.

RESULTS AND DISCUSSION

The e.s.r. spectrum of spin-labelled salt-soluble rat-skin collagen $\{\alpha(I)\}_2\alpha 2$ at pH 7.2 is shown in Figure 1. It is apparent that the spectrum is markedly different from that of the free spin label (I) under similar conditions and approximates the spectra of labelled poly-L-lysine, labelled bovine serum albumin at low pH, and other labelled proteins free of sulphydryl groups.[25] The general shape of the spectrum indicates that the nitroxide group still has considerable free motion when covalently bound to salt-soluble collagen. This suggests that there is little or no inner core binding which would result in a strongly immobilised radical.[25,31]

With hydrated fibrous collagen preparations substantially greater immobilisation of the nitroxide groups was observed consistent with the change in structural properties of the aggregated molecules. In fact the e.s.r. spectra of spin-labelled rat skin measured at 25 °C, hydrated fibrils and solid fibrils are similar to those reported for a spin probe immersed in chemically modified and pronase-treated cattle-collagen films. In both cases,

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composite anisotropic spectra are observed. For salt-soluble spin-labelled rat-skin collagen, however, only a narrow triplet was evident over the temperature range examined. These observations would suggest that insoluble collagen behaves as a bi-phasic system. A possible explanation has been advanced by Nagaimura and Woodward to accommodate the co-existence of a narrow triplet along with a broad triplet in collagen films. These

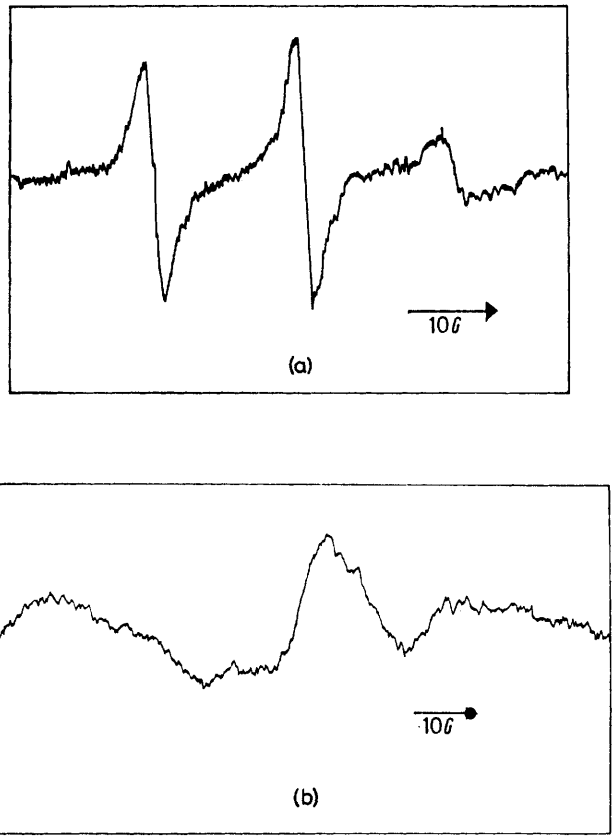

FIGURE 1 E.s.r. spectrum of salt-soluble rat-skin collagen $\{\alpha 1(I)\}_2\alpha_2$ spin labelled in 10% NaCl–phosphate buffer, pH 7.2, with the iodo-acetate spin label (I) at 25 °C. Instrumental settings were: microwave power 5 mW, gain 2×10^4, time constant 3.0 s, modulation amplitude 1.6 G. (b) E.s.r. spectrum of hydrated fibrous collagen $\{\alpha 1(I)\}_2\alpha_2$ spin labelled with the iodo-acetate spin label (I). Instrument settings were: microwave power 5 mW, gain 2×10^4, time constant 3.0 s, modulation amplitude 0. 8 G

workers conclude that one phase corresponds to the polymer with closely associated structural water and the other involves ' mobile ' water. Such an interpretation would imply that spin-labelled collagens in solution should have a spectral width and shape similar to the probe molecule in water at similar temperatures. The fact that only a triplet is evident in the e.s.r. spectra of labelled salt-soluble collagens is thus consistent with these workers' proposal.

If it is assumed that rat skin $\{\alpha 1(I)\}_2\alpha_2$ collagen behaves in solution as a prolate ellipsoid of revolution undergoing rotational diffusion, then the correlation time of rotation τ_c of an immobile radical can be calculated [25] from the Stokes–Einstein equation,

$$\tau_c = \frac{4\pi\eta_e^3}{3RT}$$

where τ_c is the correlation time, η solution viscosity, r_e the species radius, R the Boltzmann constant, and T the absolute temperature (K). For an elliptical collagen molecule ($r_{11} = 290$ nm, $r = 14$ nm),[32] the predicted τ_c for a rigidly bound spin label is of the order 10^{-2} s. At 25 °C, the observed τ_c of the labelled tropocollagen is of the order 10^{-9} s [*cf.* free spin label 10^{-11} s (ref. 25)]. Spin-labelled biomolecules generally show a marked divergence between the observed and predicted value of τ_c. This variation is usually accommodated in terms of the ' rotation in a cage ' hypothesis.[33] In this regard collagen does not appear to be exceptional. Since the spin-label method is dependent on the incorporation of a conformationally sensitive probe at a specific site, the observed spectral changes reflect more heavily the local conformational changes in the vicinity of the spin label rather than changes in the protein as a whole. The data presented below will thus relate to transitions within the micro-environment of the spin label.

For this analysis, it was important to confirm that the spin label (I) was, in fact, covalently, rather than adsorptively, attached to the macromolecule. This was achieved by examining the e.s.r. spectra of 2,2,6,6-tetramethyl-4-aminopiperidine 1-oxyl (II), a spin label probe which can undergo ionic but not covalent interaction in the presence of soluble $\{\alpha 1(I)\}_2\alpha_2$ collagen. As expected, these spectra were completely different from those obtained with the alkylating label (I) coupled to collagen and indeed, they were similar to that found for the free spin label (II) alone in distilled water. It was possible to calculate the extent of labelling by comparing the area under the absorption curves with that for a known number of free nitroxide radicals. The extent of labelling was found to be *ca.* 0.3 label per tropocollagen molecule. This low figure possibly is not due to incomplete alkylation by the spin label, but rather may indicate a selective reaction due to the aggregation of collagen under the spin labelling conditions. Since neutral salt-soluble rat-skin $\{\alpha 1(I)\}_2\alpha_2$ collagen used here is believed to be devoid of free sulphydryl groups,[34] the alkylation reaction has presumably occurred on available amino-groups. As commented on above, the spectral data is consistent with the interpretation that these sites, possibly the exposed ε-NH_2 groups of lysine residues which are radially disposed on the cylindrical surface of the elongated molecule,[14] are on the extrinsic surface of the tropocollagen molecule and exposed to the solvent. This selective surface labelling of tropocollagen is analogous to that experienced with other proteins which either lack or have blocked SH groups.[31] Degradative

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studies are under investigation to define the specific site of labelling.

The covalently spin-labelled collagen exhibited a series of temperature-induced transitions which parallel those

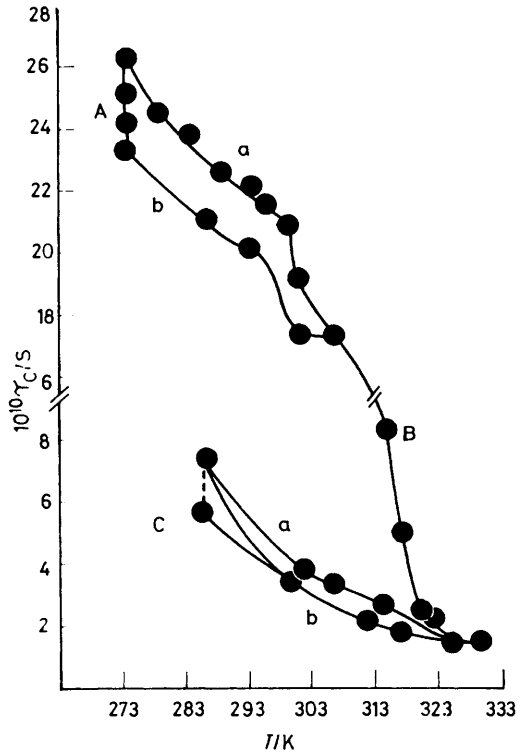

FIGURE 2 Dependence of the spin-label correlation time (τ_c) upon absolute temperature for the spin-labelled salt-soluble rat-skin collagen in 10% NaCl–phosphate buffer, pH 7.2. The upper hysteresis curve (A) follows the reversible conformational changes as neutral salt-soluble collagen gels on increasing the temperature (a) and remelts on decreasing the temperature (b). The curve (B) corresponds to the soluble collagen–parent gelatin transition. The lower hysteresis curve (C) follows the reversible conformational changes of parent gelatin for (a) increasing and (b) decreasing temperatures. The dashed region represents the equilibration of a sample rapidly cooled from 30 °C to 10 °C and held at that temperature for 12 h. After that time, the spectrum was again recorded and τ_c recalculated

observed for native material. The first relates to the reversible conformational changes as neutral salt-soluble collagen gels. A plot of τ_c against temperature over the range 5—25 °C for both increasing and decreasing temperatures revealed a regular hysteretic phenomenon (Figure 2 upper curve). These changes may be associated with the inhibited movements of the polypeptide chains in collagen as they fluctuate between several positions of equilibrium coupled with the phase change which appeared to occur near 20 °C. At this temperature, a reproducible increase in mobility of the label was observed. On increasing the temperature above 25 °C, the mobility of the spin label undergoes a further moderate increase. A major change in mobility occurred in the range 39—40 °C which corresponds to the soluble collagen–parent gelatin transition. The effect observed is consistent with the model involving a spin-label site that suddenly exhibits much less restriction to the tumbling of the probe. On cooling the parent gelatin, a second hysteresis effect became apparent in the spectra. The mobility of the spin label does not, however, decrease to that of the original labelled collagen sample at the same temperature (Figure 2 lower curve). This divergence indicates that incomplete refolding occurs and that the transformation is not strictly a two-state reversible process. Concordant with this observation are results based on polarimetric and o.r.d. methods [14,16] which have also detected conformational nonequivalence between refolded parent gelatin and tropocollagen. The characteristic melting point shapes shown in Figure 2 are reminiscent of those obtained by other methods. von Hippel and Wang,[35] and more recently Russell [14] have reported transition temperature widths of $\Delta T = 2 \pm 0.5$ °C for native collagen based on polarimetric measurements on dilute solutions. The transition temperature for the unfolding process, $T_m = 40$ °C (±1 °C), and the transition width, $\Delta T = 4$ °C (±1 °C), obtained in the present study with the labelled collagen are thus in good agreement with reported values. Furthermore, this suggests that the alkylation treatment has not affected the intra- or inter-chain alignment or modified to any substantial effect the native conformation.

The activation energies for the various stages of the transitions may be determined from a plot of $\ln 1/\tau$ *vs.* $1/T$ The slopes of the linear portions give estimates of the activation energies which relate to the processes of expansion of the environment about the spin label and

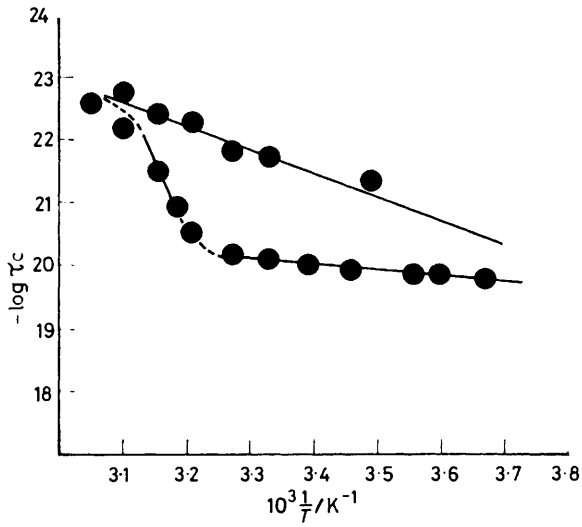

FIGURE 3 Arrhenius plot of the spin-labelled salt-soluble rat-skin collagen. The correlation times (τ_c) were calculated using the method of Stone *et al.*[25] The lines of best fit were generated by the least-squares analysis of all the data points. The dashed portions represent the transition regions

hence increases in mobility.[23] From the Arrhenius plot (Figure 3) three discrete processes are evident. The first, covering the temperature range 5—34 °C with an

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apparent $E_a = 8 \pm 1$ kJ mol^{-1} clearly is a measure of the weak, reversibly intermolecular changes arising from oligomer aggregation–disaggregation and solvent interaction with exterior sites of the tropocollagen molecules. A similar effect has also been observed with bovine ribonuclease A.[36] This gradual mobilisation of the spin label on the tropocollagen undergoes a sudden crossover in the range 34—49 °C, centred about the transition temperature T_m, which indicates that a second major step in the expansion of the region surrounding the spin-probe has come into play with a substantially higher activation energy of $E_a = 108 \pm 30$ kJ mol^{-1}. This second process, which corresponds to the unwinding of the macromolecular tropocollagen into random coils, can not be accounted for solely in terms of a marked decrease in both intra- and inter-chain ionic or London force interactions. The activation energies associated with the tropocollagen→gelatin transition can be considered as a composite term arising from the kinetic processes involved in the rupture of inter- and intra-chain hydrogen bonds, the cleavage and re-alignment of interchain hydrophobic interactions and the collapse of the water structure near the macromolecule. Based on their studies of the tropocollagen→gelatin transition in soluble collagens of widely different amino-acid content, Privalov and Tiktopulo[37] conclude that the regular water structure associated with the macromolecule played an important role in stabilising the tropocollagen. Recent evidence from n.m.r. and differential scanning calorimetric studies[38] supports this conclusion. The observed high activation energy indicates that the immediate environment of the spin label is undergoing a substantial expansion. Although it is not possible from the present study to estimate the contributions each of these effects makes to the unwinding phenomenon, it is unlikely that the large activation energy for the expansion process is solely due to the breaking of strong hydrogen bonds. Recently, Menashi and his co-workers[38] reached a similar conclusion based on enthalpy data associated with the denaturation of collagens of different amino-acid content.

The smaller activation energy ($E_a = 35 \pm 3$ kJ mol^{-1}), observed for the third process corresponds to folding and unfolding of the parent gelatin. The fact that τ_c for gelatin formed in this cycle does not become coincident with τ_c for collagen at the same temperature (τ_c collagen $> \tau_c$ gelatin in the range 0—40 °C) indicates that not all the conformational perturbations which collagen can undergo on melting are re-established in gelatin.

Complementary charge interactions exert a considerable influence in stabilising the rigid helical structure of tropocollagens.[39] At low or high pH, particularly in the presence of anions with chaotropic or lyotropic activity, strong charge repulsions develop. These disrupt the intrinsic stability of the molecules and result in rigid helical form→random coil-like transitions. It thus was of interest to examine the effect of pH and dissociating reagents on the mobility of the spin label (see Table). Addition of urea in increments such as to make its concentration 1M, 2.5M, and 5M had significant effects on the e.s.r. spectrum of the labelled tropocollagen with the spin label being considerably more mobile at the higher concentration. A similar trend was also observed with guanidine hydrochloride. The presence of either 1M-urea or 0.67M-guanidine hydrochloride resulted in spectra intermediate between those of the soluble tropocollagen and the parent gelatin presumably due to the partial destabilisation of the triple helix. At a concentration of 5M, both dissociating reagents resulted in a further increase in the mobility of the spin label on tropocollagen, which approached that observed in the random coil situation, *e.g.* parent gelatin at a similar temperature. These observations demonstrate that the immediate environment of the spin probe undergoes a considerable expansion in the presence of urea or guanidine hydrochloride similar to that observed in the thermal transition. These changes in the probe mobility as the tropocollagen molecule reversibly unwinds correlate well with results obtained with related experiments run in the presence of 0.5M-acetic acid. Under these conditions, complementary charge interactions are minimised due to protonation of the carboxy-groups. This will result in strong charge repulsions developing within the helix leading to destabilisation. Significant increases in mobility of the spin probe are also seen under these conditions (Table). This variation in the probe mobility as the tropocollagen unfolds appear to reflect the intrinsic changes of the whole molecule since similar results have also been obtained by polarimetry.[14]

Temperature dependence of correlation time for spin-labelled salt-soluble rat-skin collagen in the presence of various reagents

Spin-labelled collagen	$10^{10}\tau_c$/s	T/K
1M-Urea	17.31	279.5
1M-Urea	13.59	293.5
2.5M-Urea	13.19	294.5
5M-Urea	9.21	294.5
0.67M-GdCl	15.5	286.5
3.33M-GdCl	6.84	286.5
0.5M-Acetic acid	11.24	279.5
0.5M-Acetic acid	2.65	307.0
Bacterial collagenase * (0.5 mg ml^{-1})	11.32	273.0
Bacterial collagenase * (0.5 mg ml^{-1})	3.41	293.5
Bacterial collagenase * (0.5 mg ml^{-1})	1.57	307.0
Trypsin * (0.5 mg ml^{-1})	25.41	273.0
Trypsin * (0.5 mg ml^{-1})	14.11	293.5

* The spin-labelled collagen was incubated with the bacterial collagenase for 5 min. and trypsin for 1 h at ambient temperature (*ca.* 18 °C).

Bacterial collagenase cleaves native collagens at multiple loci producing fragments still in the helical conformation.[39,40] The generation of these fragments from a spin-labelled tropocollagen should be associated

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with a substantial increase in the mobility of the probe. This, in fact, was observed with τ_c progressively decreasing for temperatures in the range 0—34 °C and an enzyme concentration of 0.5 mg ml^{-1} (Table). At 0—5 °C, the digestion with bacterial collagenase proceed at a rate sufficiently low for the kinetics of cleavage to be measurable.[41] Addition of trypsin has little effect on the e.s.r. spectrum of the labelled tropocollagen (Table). The only detectable effect was a slight broadening of all the spectral lines. Since this enzyme is known [42] to cleave initially the non-helical teleopeptides from the triple helix, this result suggests that the spin labelling has, in fact, occurred at site(s) in the helical domains. It should be also noted that dialysis of the bacterial collagenase treated spin-labelled collagen demonstrated that covalently bound spin-labelled polypeptide fragments passed through a membrane with a nominal molecular weight cut-off of 6 000 Daltons. The corresponding trypsin-treated material was retained.

[41] M. T. W. Hearn, D. B. Myers, and B. M. Peake, unpublished observations.

In summary, e.s.r. studies of spin-labelled $\{\alpha 1(I)\}_2\alpha_2$ rat-skin collagen permit a more detailed picture of the thermal denaturation of native salt-soluble collagen to be obtained. Based on the examination of the spectral changes, it is apparent that the micro-environment around the spin label undergoes a series of hysteretic changes which reflect those observed for the whole molecule. The activation energies calculated from the Arrhenius plots provide an indication of the energetics of the expansion of the micro-environment about the labelled site. An important question now to be resolved is the extent to which this reflects the behaviour of the entire tropocollagen macromolecule.

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