RELAXATION BEHAVIOR OF COLLAGEN

H. STEFANOU, A. E. WOODWARD, *and* DARRELL MORROW

From the Department of Chemistry, The City College of The City University of New York, New York 10031 and Department of Mechanics and Materials Science, Rutgers University, New Brunswick, New Jersey 08903

ABSTRACT The dynamic mechanical properties of purified collagen from bovine tendon were studied using a torsion pendulum in the temperature range of 120°–360°K at 0.3–1 cps. In the temperature range studied, two loss peaks were observed: a β-peak at about 200°K, and an α-peak approximately five times larger at about 280°K. The temperature of the α-transition is shown to be dependent on water content, decreasing with increasing amount of water and shifting to lower temperatures. Broad-line proton magnetic resonance results were also obtained on similar samples. A narrow nuclear magnetic resonance (NMR) line appears at about 250°C. The effects of shrinkage to form gelatin and of cross-linking on the relaxation behavior of collagen were also studied. The motions taking place in collagen over the 120°–360°K range are discussed.

INTRODUCTION

Since collagen serves as the chief load-bearing (tensile) element for all mammals and fish, a considerable literature exists on studies of this material (1). The thermal contraction process in heated collagen fibers to form gelatin, believed to involve crystalline melting, has been followed using both kinetic (2–4) and thermodynamic (5–9) approaches. Static mechanical measurements on preshrunk collagen, swollen with various liquids, have been used to estimate the number of cross-links (10–13).

Some studies of the relaxation behavior of collagen and of gelatin at low to moderate temperatures have been reported to date. Proton and deuteron NMR studies have been carried out on rat tail tendon collagen in the 220°–315°K region with the behavior of the narrow line being of principal interest (14–16). Dielectric studies of collagen (17) and various gelatins (18) have been described. Commercial gelatin preparations have been the subject of two dynamic mechanical investigations (19, 20). Dynamic mechanical investigations on collagens of various origins have been recently reported (21–23).

In the present work the relaxation behavior of purified collagen from steer tendon has been investigated by two methods. The dynamic shear behavior at 1–10 cps was studied in the 90°–360°K temperature range using a torsion pendulum; broad-line NMR spectra at 90 mHz were obtained in the 180°–340°K range. Two mechanical relaxation processes were evident along with the appearance of a narrow NMR

line. The effects of water, of shrinkage to give gelatin, and of formaldehyde cross-linking on the relaxation behavior were also investigated. It is concluded that at room temperature both side-chain reorientations and main-chain oscillations are taking place in collagen and gelatin.

EXPERIMENTAL

The collagen used in this study was obtained from Johnson and Johnson Co. as a 2% aqueous suspension. The fibrils in the suspension are reported to be of uniform diameter and length and are taken from steer heel tendon by a process which includes chopping and removal of extraneous protein (24). Sheets of the collagen fibrils were prepared by pouring the suspension onto a plastic plate and allowing it to solidify in air at room temperature, thicknesses of 50–200 μm $\pm$ 10 μm being obtained. These sheets were stored at room conditions for 2 months or more before further use. Some samples were dried under vacuum at room temperature to constant weight; the water contents referred to in the next section are relative to the water content of a sample dried in this way. A further weight loss of 27% occurred when such a specimen was heated for 30 min at 160°C; this weight loss is in agreement with a value given by Heideman and Keller (25). Water contents of the samples used in the dynamic mechanical and NMR measurements were achieved by using samples equilibrated above salt solutions or in the laboratory. The water contents cited were those existing in the sample after a dynamic mechanical or NMR run was made. Except for samples dried under vacuum at room temperatures, the specimens used were not heated above 305°K during any runs for which data are given in the next section. No detectable orientation in the collagen film as prepared could be found by an X-ray diffraction experiment with the beam perpendicular to the film. The cast collagen films were found to have about a 4% water-soluble portion.

Specimens of gelatin (shrunken collagen) for NMR measurements were prepared by heating collagen strips immersed in water to 80°C. Upon cooling back to room temperature, it was found that about 60% shrinkage in length of the strip took place. Specimens for the dynamic mechanical studies were prepared by heating the collagen dispersion in water to 100°C and then casting to form strips.

Cross-linking of collagen was carried out by immersing a strip in 10% formaldehyde solution at pH 8 for 3 h. The swelling of part of this sample after heat denaturation was followed using ethylene glycol as the swelling agent. A non-cross-linked denatured sample was used as a control. The amount of ethylene glycol imbibed at equilibrium was 1.5 g/g of polymer for the cross-linked sample and 2.8 g/g polymer for the control.

The dynamic shear properties were measured using a torsion pendulum apparatus designed by Sinnott (26) and modified by Frosini and Woodward (27). Strips of 1.0 cm width and from 5 to 7 cm length were cut from the cast sheets. Tensile loads of 25, 48, and 83 g were used.

NMR measurements were carried out at 90 mHz using a Brücker spectrometer. Although attempts were made to avoid modulation broadening, the narrow line widths measured may be too wide by an unknown amount due to this effect.

RESULTS

Some results obtained for collagen samples of different water contents using the torsion pendulum are given in Fig. 1 in terms of the damping (logarithmic decrement) and the real part of the shear modulus G'. It is seen that at about 1 cps in the

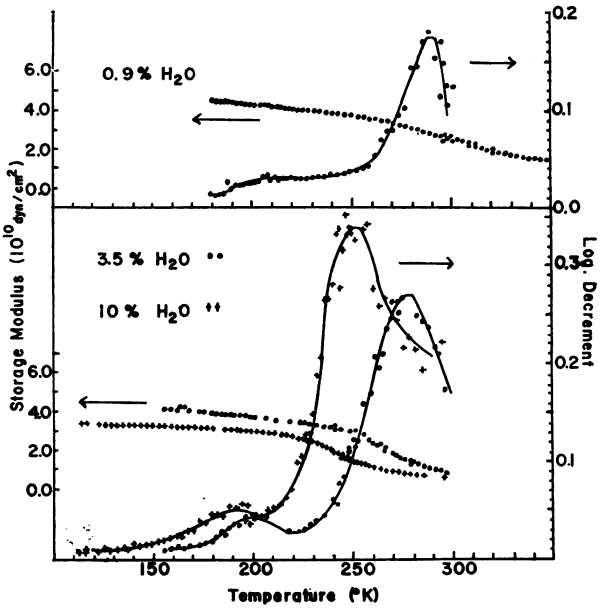

FIGURE 1 Dynamic shear behavior of steer tendon collagen at 0.3–1 cps as a function of water content and temperature.

100–300°K temperature range there are two loss processes. The α-process (~290°K for a vacuum-dried sample) is a major one with at least a twofold drop in storage modulus taking place with increasing temperature. This process shifts to lower temperatures and increases in intensity with increasing water content. The β-process at about 200°K also shifts to lower temperatures with increasing water content. The height of the β-loss maximum increases somewhat with water content.

The dynamic shear behavior for a shrunken collagen sample dried under vacuum at room temperature and for another sample containing 10% water are shown in Fig. 2. The principal difference between shrunken and parent material is the twofold increase in storage modulus at low temperatures upon shrinkage.

The effects of formaldehyde cross-linking on the dynamic mechanical behavior for a water-containing specimen is shown in Fig. 3. It can be seen that cross-linking markedly decreases the α-loss peak and shifts the β-process by about 30–40° to lower temperatures. In addition, the modulus is increased by at least twofold at low temperatures (120°K). The modulus near 300°K drops suddenly with increasing temperature for the cross-linked sample, then increases. This change was found to be reversible and independent of the sample's water content.

Broad-line NMR measurements were carried out in the 180°–380°K region on a vacuum-dried collagen sample and a sample with 6.5% water added. Below about 240°K a single line of 8–9 G width was evident; around 260°K a very narrow line appeared, the width of it being less than 0.1 G. At the lowest modulation ampli-

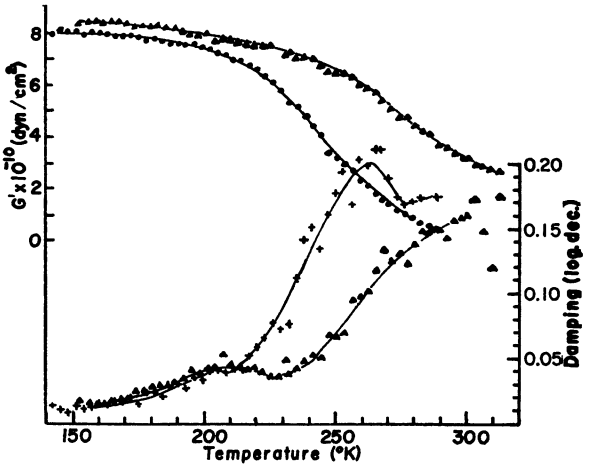

FIGURE 2 Dynamic shear behavior of shrunken collagen at 0.3–1 cps. ▲, dry; + and ●, 10% H_2O.

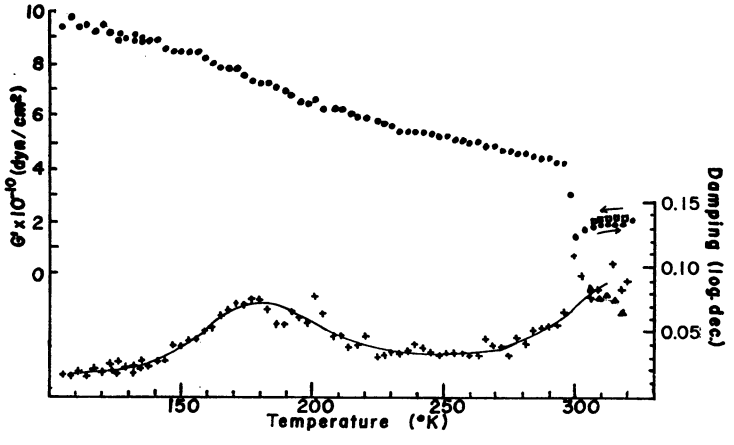

FIGURE 3 Dynamic shear behavior of formaldehyde cross-linked collagen (5½% H_2O).

tudes possible, the narrow line was 0.04 G at 305°K. The appearance of this narrow line takes place at temperatures definitely below those at which the mechanical process at 1 Hz is found. Assuming that the mechanical loss peak is due to a relaxation process, any changes in the NMR associated with it should be at higher temperatures, since the frequency for an NMR process is 10^4 Hz or greater. Therefore, the initial appearance of the narrow line can not be associated with the mechanical α-process. The fact that the temperature of first appearance of this line does not depend on the water content is further evidence for this.

For the vacuum-dried collagen sample the NMR absorption shows a steady drop in second moment with increasing temperature over the total range studied, as can

be seen in Fig. 4. The presence of 7% water causes the second moment drop to become more precipitous in certain regions of temperature. The process in the 260° to $\geq$300°K region for this sample can be tentatively correlated with the α-loss maximum at 260°K (1 cps) for a sample of the same water content.

Vacuum-dried shrunken collagen also shows a narrow line which first becomes evident at about 250°K. Extended drying under vacuum at 373°–380°K brought about no change in this line at 300°K. For a sample containing 8½% water the narrow line width was 0.4 G at 253°K changing to 0.2 G at 265°K and above.

The second moment in the 200°–350°K region for a vacuum-dried sample, plotted in Fig. 5, shows a more precipitous drop than that found for collagen. The effect of added water on the NMR second moment results is to split the gradual drop into a relatively sharp process at 210°–260°K and a more gradual one at 260°K and up. This latter process has a lower slope than that for collagen in the same temperature

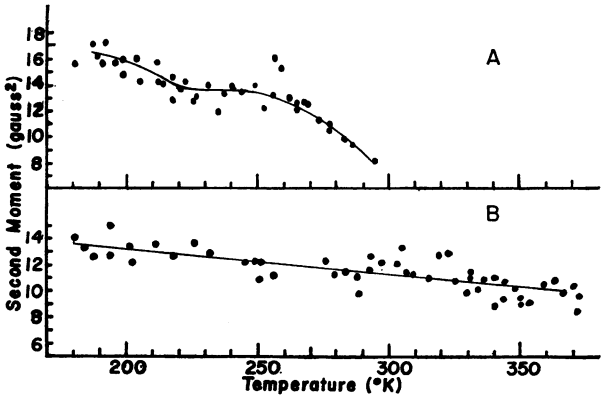

FIGURE 4 NMR second moment vs. temperature for collagen with different water contents. (A) 6½% H_2O; (B) dry.

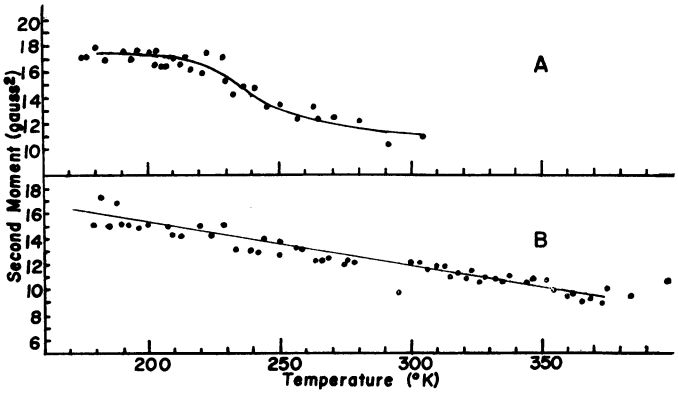

FIGURE 5 NMR second moment vs. temperature for shrunken collagen. (A) 8½% H_2O; (B) dry.

region. It is doubtful that the 210°–260°K NMR process or the first appearance of the narrow line are related to the mechanical α-peak. The gradual NMR change above 260°K could, however, be related to the α-process.

DISCUSSION

From a torsion pendulum study of 30-yr-old human diaphragm collagen, Papir et al. (22) reported three loss peaks, two of which appear to correspond to the α- and β-peaks seen in Fig. 1, and a third at higher temperatures. However, the peak found by Papir et al. which corresponds to the α-process in temperature position (see Fig. 1) does not show a shift with water content, and for a dry specimen is smaller than that shown in Fig. 1; in addition, the peak for a dry specimen was reported at about 150°K. It should be noted that those results are more in agreement with those found in this study for formaldehyde cross-linked material than for the parent collagen. Therefore, it is possible that due to the age of the sample used by Papir et al. (22), it had a much higher degree of cross-linking than the collagen used in this study.

Chien and Chang (23) recently reported a sizable α-peak for moist rat tail tendon and two lesser processes at -100°C and -50°C, respectively, presumably at 110 Hz. On the other hand, an enzyme-solubilized collagen sample showed only two small peaks. Again the differences between the present results and those of Chien and Chang (23) may arise due to real differences in samples.

NMR measurements were previously reported on oriented rat tail tendon in water and D_2O with the narrow line being of principal interest (14–16). It was found (16) that the narrow component decreases markedly in intensity in the 270°–220°K region as compared to 250°K found in the present work for an unoriented sample. It was also shown earlier (16) that some of the protons which contribute to the narrow component do not exchange with D_2O. An explanation put forth was that relaxation of —OH protons on the hydroxyproline residues was taking place, assuming that these groups are in the interior of the collagen triple helix and not available to exchange with H_2O or D_2O. There is no evidence in the present study which disproves this assignment. The protons on the flexible paraffinic portions of the side chains would also be nonexchangeable and therefore could be responsible for part of the narrow line. However, if that were the case, then a mechanical loss process would be expected to be correlated with the narrow line. The only loss peak in a suitable frequency-temperature position is the β-peak at $\sim$200°K (1 Hz). However, from results obtained for various synthetic polypeptides with flexible R groups, this temperature appears to be too low for motion of these groups.

The flexible side chains present in collagen and gelatin include the R groups of lysine, aspartic acid, and glutamic acid. Synthetic polypeptides made from derivatives of lysine (28) and glutamic acid (29) are known to show mechanical loss peak at 260°–300°K (0.2–1 Hz). Copolymers of L-glutamic acid and L-leucine have loss

peaks at about 270°K (1 cps) (30). Poly(γ-benzyl-L-glutamate) exhibits (31) a decrease in second moment to a value near zero somewhat above 300°K; poly(sodium α-L-glutamate) containing traces of water shows a narrow line at temperatures as low as 260°K. Therefore, it seems more reasonable to ascribe the α-mechanical loss peak and the associated changes in the NMR second moment to side-chain reorientation.

The effects of cross-linking and of the presence of water on the relaxation behavior appear to be consistent with the above assignment for the α-process. Crosslinking with formaldehyde is expected, at least if the conditions are kept mild enough, to involve only the amine groups at the chain ends or on the basic side chains (1). This reaction should give greater rigidity to the segments connected to these functional groups and this should cause an increase in modulus. It is expected that any loss process involving these segments would be interfered with. Therefore, the mechanical behavior of the crosslinked collagen is consistent with the assignment of the α-process to side-chain motion. The lowering of the α-peak temperature with increasing water content for collagen and gelatin could possibly be due to the breaking up of acid-base side-chain associations, which would lead to lower barriers to reorientation. It is expected that the associated water molecules would also be reorienting with the side chains, thereby increasing the size of the α-peak.

Assuming that the above explanation for the α-process is correct, an assignment for the β-process remains to be made. It was recently shown[1] that poly(L-proline) in the II conformation shows a relaxation process at 140°–230°K (0.4 Hz) and 180°–280°K (10^4 Hz). This was attributed to oscillations of the proline groups about the main-chain within the rather broad energy well predicted for form II (32). It is known that collagen contains large amounts of proline and hydroxyproline residues, and it is believed that the individual collagen chains in the super helix are in a poly(L-proline) II-like helix (1). Therefore, it seems reasonable to assume that some low frequency main-chain oscillations could be taking place in collagen in the temperature region in which the β-process occurs and are, therefore, mainly responsible for this process.

Dehl and Hoeve (16) reported that the reorientation of water molecules in oriented rat tail tendon can take place at temperatures as low as 220°K (10^4 Hz), and Hiltner and coworkers (30) reported small loss maxima at about 175°K (1 Hz) due to water in synthetic polypeptides. It is possible, therefore, that at least part of the drop in second moment in water containing-collagen and -gelatin around 200°K is due to reorientation of bound water molecules.

The present dynamic mechanical results show that the change from collagen to gelatin leads to greater rigidity. This suggests that gelatin has a tighter structure than collagen, despite the fact that the former is considered to be more amorphous.

This investigation was partially supported by Biomedical Sciences support grant U. S. Public Health

[1] Stefanou, H., and A. E. Woodward. Unpublished results.

Service 1S05RR07131-01 from the National Institutes of Health and by the New York State Foundation for Science and Technology.

Received for publication 15 December 1972.

REFERENCES

1. VEIS, A. 1964. *Mol. Biol.* **5**.
2. WEIR, C. E. 1949. *J. Am. Leather Chem. Assoc.* **44**:108.
3. WEIR, C. E., and J. CARTER. 1950. *J. Res. Natl. Bur. Stand.* **44**:599.
4. CREWTHER, W. G., and L. M. DOWLING. 1958. *J. Physiol. Chem.* **62**:681.
5. KUNTZEL, A., and DOEHNER, K. 1939. *Angew. Chem. Int. Ed. Engl.* **52**:175.
6. WOHLISCH, E. 1932. *Biochem. Z.* **247**:329.
7. WOHLISCH, E. 1939. *Kolloid Z.* **89**:239.
8. WOHLISCH, E., H. WEITNAUER, W. GRÜNING, and R. ROHRBACH. 1943. *Kolloid Z.* **104**:14.
9. FLORY, P. J., and R. R. GARRETT. 1958. *J. Am. Chem. Soc.* **80**:4836.
10. WIEDERHORN, N. M., and G. V. REARDON. 1952. *J. Polym. Sci.* **9**:315.
11. KULONEN, E., U. K. VIRTANEN, and A. SALMENPIRA. 1962. *Acta Chem. Scand.* **16**:1579.
12. CATER, C. W. 1963. *J. Soc. Leather Trades' Chem.* **47**:259.
13. ELDEN, H. R. 1969. *Trans. N. Y. Acad. Sci.* **31**:855.
14. BERENDSEN, H. J. C. 1962. *J. Chem. Phys.* **36**:3297.
15. BERENDSEN, H. J. C., and C. MIGEHELSEN. 1965. *Ann. N. Y. Acad. Sci.* **125**:365.
16. DEHL, R. E., and C. A. J. HOEVE. 1969. *J. Chem. Phys.* **50**:3245.
17. LIM, J. L., and M. H. SHAMOS. 1971. *Biophys. J.* **11**:648.
18. SAIKI, K., and Y. OKAMOTO. 1966. *Jap. J. Appl. Phys.* **5**:962.
19. KOLASKE, J. V., and J. A. FAUCHER. 1965. *J. Phys. Chem.* **69**:4040.
20. GILLHAM, J. K. 1966. *Appl. Polym. Symp.* **2**:45.
21. MASON, P., and J. UNSWORTH. 1971. *Kolloid Z. Z. Polym.* **249**:1101.
22. BAER, E., R. KOHN, and Y. S. PAPIR. 1972. *J. Macromol. Sci. Phys.* **B6**:761.
23. CHIEN, J. C. W., and D. E. P. CHANG. 1972. *Biopolymers.* **11**:2015.
24. LIEBERMAN, E. R. 1971. Studies of the permeation of gases through collagen films. Ph.D. Thesis. Rutgers University, New Brunswick, N. J.
25. HEIDEMAN, E., and A. KELLER. 1970. *J. Am. Leather Chem. Assoc.* **65**:512.
26. SINNOTT, K. M. 1958. *J. Appl. Phys.* **29**:1433.
27. FROSINI, V., and A. E. WOODWARD. 1969. *J. Macromol. Sci. Phys.* **B3**:91.
28. HILTNER, A., J. M. ANDERSON, and E. BORKOWSKI. 1972. *Macromolecules.* **5**:446.
29. SABA, R. G., J. A. SAUER, and A. E. WOODWARD. 1963. *J. Polym. Sci.* **A1**:1483.
30. HILTNER, A., E. BAER, and J. M. ANDERSON. 1972. *Polym. Prepr.* **13**:1147.
31. KAIL, J. A. E., J. A. SAUER, and A. E. WOODWARD. 1962. *J. Phys. Chem.* **66**:1292.
32. DESANTIS, P., E. GIGLIO, A. M. LIQUORI, and A. RIPAMONTI. 1965. *Nature (Lond.).* **206**:456.