BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 2890—2894 (1967)

Soluble Collagen of Chicken Leg Tendon; Its Denaturation Temperature and Hydrodynamic Properties

Rume SAKAI, Shoichi IKEDA and Toshizo ISEMURA

Institute for Protein Research and Faculty of Science, Osaka University, Osaka and Toyonaka, Osaka

(Received June 2, 1967)

The soluble collagen was extracted from chicken leg tendon with a 0.15 M citrate buffer at pH 3.8. The denaturation temperature was estimated to be 38°C by measuring the intrinsic viscosity and also by measuring the optical rotation at 589 mμ. The optical rotatory dispersion constants (λ_c) of collagen and gelatin were found to be 210±10 and 220±10 mμ respectively. The intrinsic viscosity of collagen was 12.5±1.0 dl/g. The rotational diffusion coefficient at 20°C in water was 795±75 sec^{-1}. The sedimentation constant at 20°C in water was 3.0S. From these data, the size and molecular weight of collagen were discussed. The collagen of chicken leg tendon is 2900 to 3000 Å in length and 12 to 16 Å in diameter, and has a molecular weight of 34 $\times 10^4$.

The soluble collagen was extracted from collagen fiber under mild conditions.[1] From this solution, the fiber can be reconstituted; this fiber has been demonstrated to have the same appearance as the original fiber when investigated by microscopy,[2] electron microscopy[3] and X-ray diffraction.[4] On the basis of these facts, the physicochemical properties of the soluble collagen in solution have been extensively studied. The soluble collagen has been reported to be polydisperse or a material containing molecular species of a different molecular weight depending on the source.[5] In 1956 Boedtker and Doty[6] reported that the ichthyocol of carp swim bladder was a rod-like molecule 3000 Å in length and 13.6 Å in diameter, and with a molecular weight of 345000. The other soluble collagens reported thereafter have almost the same molecular weight.[5]

If the temperature of a solution of collagen is gradually raised, a very sharp transition of such properties as the optical rotation and the viscosity is observed at a certain temperature.[6,7] This phenomenon may be attributed to the fact that collagen stable at lower temperature changes to gelatin at the temperature called the denaturation temperature, the point of which depends on the species of collagen.

Although the temperature dependence of the properties of the collagens obtained from various kinds of animals has been investigated, that of the collagen of avian tendon, easily obtained in a very pure state, has not yet been thoroughly studied.

In the present investigation, the soluble collagen of chicken leg tendon was studied. The denaturation temperature was determined by measurements of the viscosity and the optical rotation. The size and molecular weight of the collagen were discussed by the examining such hydrodynamic properties as viscosity, sedimentation and flow birefringence.

Experimental

The soluble collagen was prepared in the following way from chicken leg tendon. Chicken tendon freed from meat was minced and soaked in 0.5 M sodium acetate to remove any soluble proteins and carbohydrates. The tendon was then placed in a 0.15 M citrate buffer at pH 3.8 in order to extract the crude soluble collagen. The extract was filtered and dialysed against 0.02 M disodium hydrogen phosphate. The white fibrous collagen which appeared in the dialysis-bag was collected and washed with water. The collagen thus obtained was redissolved in the citrate buffer, and precipitated by dialysis for further purification. The soluble collagen was then stored in a deep freezer. The solubility in the buffer was less than 0.1% below pH 5. When the solution was kept for about two weeks in a refrigerator, some precipitates separated. A sample solution of the collagen was prepared as follows: the stored collagen was suspended in a 0.15 M citrate buffer at pH 3.8. The suspension was then stirred in a cold room for about three days and dialysed against the same buffer, after which the dialysed solution was centrifuged at 80000 g for two hours.

1) P. A. Zachariades, *Compt. rend. soc. biol.*, **52**, 182, 251, 1127 (1900).
2) J. Nageatte, *Compt. rend. acad. sci.*, **184**, 115 (1927); *Compt. rend. soc. biol.*, **96**, 172 (1927).
3) F. O. Schmitt, C. E. Hall and M. A. Jakus, *J. Cellular Comp. Physiol.*, **20**, 11 (1942).
4) R. W. G. Wyckoff and R. B. Corey, *Proc. Soc. Exptl. Biol. Med.*, **34**, 285 (1936).
5) W. F. Harrington and P. H. von Hippel, "Advances in Protein Chemistry," XVI, Academic Press, New York (1961), p. 1.
6) H. Boedtker and P. Doty, *J. Am. Chem. Soc.*, **78**, 4267 (1956).
7) R. E. Burge and R. D. Hynes, *J. Mol. Biol.*, **1**, 155 (1959).

The concentration of the collagen in the solution was determined by the micro-Kjeldahl method. The value of 17.8% used as the nitrogen content was reported by Leach[8] for chicken tendon gelatin.

The optical rotation was measured on a Hitachi EPU-2 spectrophotometer equipped with a polarimeter attachment A-1. The temperature dependence of the specific rotation at 589 mμ was measured 30 min after every setting at certain temperatures.

The viscosity was measured with a dilute solution (concentration below 0.04%) using an Ubbelohde-type viscometer, the flow time of which was 154 sec for water, because Boedtker and Doty[6] reported that the viscosity of an ichthyocol solution in such a concentration was independent of the shearing stress.

The reduced viscosity was measured as a function of the temperature at different concentrations. As the viscosity at 30—40°C changed with time, it was measured 30 min after settings at definite temperatures.

The extinction angle (χ) was measured on a Rao Streaming Birefringence Instrument over a range of velocity gradients from 600 to 4000 sec^{-1} at 25°C. The light path of the sample chamber was 10 cm, while the wavelength of the light was 517 mμ.

The sedimentation experiment was carried out in a Hitachi Model UCA-1 type ultracentrifuge at 60000 rpm.

The shrinkage temperature of chicken tendon was measured in a water bath.

Results

Optical Rotatory Properties. The specific rotation at 589 mμ was $-380\pm20°$ below 35°C and $-130\pm20°$ above 40°C (Fig. 2). The denaturation of collagen to gelatin occurred in this temperature range. This is clearly shown in Fig. 1 by the plot of $([\alpha]_t-[\alpha]_G)/([\alpha]_C-[\alpha]_G)$ against the temperature, where $[\alpha]_t$ is the specific rotation at 589 mμ at t°C, and where $[\alpha]_C$ and $[\alpha]_G$ are those of native collagen and completely denatured gelatin respectively. The denaturation temperature was determined to be 38°C, which corresponds to the midpoint of Fig. 1. The optical rotatory dispersion curves of collagen and gelatin are shown in Fig. 2. The curve for collagen shifted to the negative side of that for gelatin. This phenomenon was absolutely different from those of ordinary proteins.[9] It is probably caused by the unfolding of the left handed helix of collagen.[10] These dispersion data obeyed the simple Drude equation. The dispersion constants (λ_c) of collagen

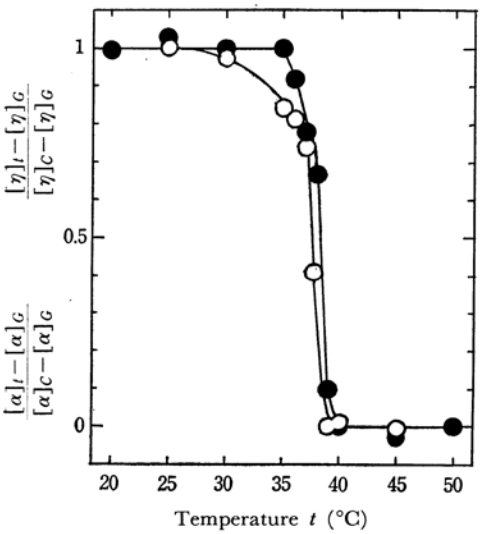

Fig. 1. The temperature dependence of specific rotation at 589 mμ and intrinsic viscosity.
$\frac{[\alpha]_t-[\alpha]_G}{[\alpha]_C-[\alpha]_G}$ ●, $\frac{[\eta]_t-[\eta]_G}{[\eta]_C-[\eta]_G}$ ○

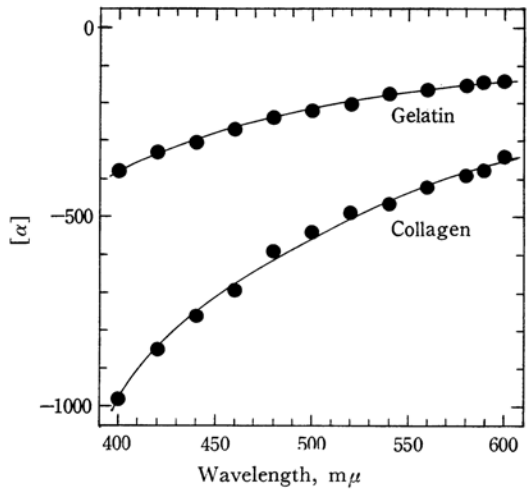

Fig. 2. Ooptical rotatory dispersion of collagen and gelatin.

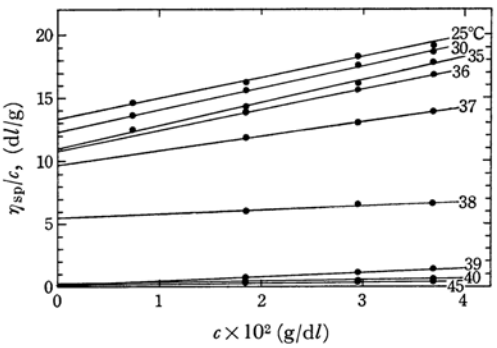

Fig. 3. Plot of reduced viscosity of collagen *versus* concentration at various temperatures.

8) A. A. Leach, *Biochem. J.*, **67**, 83 (1957).

9) P. Urnes and P. Doty, "Advances in Protein Chemistry," XVI, Academic Press, New York (1961), p. 401.

10) A. Rich and F. H. C. Crick, "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), Pergamon, London (1958), p. 20; G. N. Ramachandran, "Aspects of Protein Structure," Academic Press, New York (1963), p. 39.

and gelatin were 210±10 and 220±10 mμ respectively. They are almost equal to those of collagen and gelatin obtained from other species.[5,11]

Viscosity. The reduced viscosity curves are plotted against the concentrations of collagen at various temperatures in Fig. 3. The reduced viscosity of collagen changes linearly with the concentration. At higher temperatures the concentration dependence of the reduced viscosity decreases. The intrinsic viscosity of soluble collagen at the lower temperature of 30°C was 12.5±1.0 dl/g, which was close to those of other collagens (11—15 dl/g).[5] The intrinsic viscosity of gelatin was 0.1 to 0.4 dl/g. The effect of the temperature on the intrinsic viscosity is shown in Fig. 1 by plotting $([\eta]_t-[\eta]_G)/([\eta]_C-[\eta]_G)$ against the temperature, where $[\eta]_t$ is the intrinsic viscosity at t°C, and $[\eta]_C$ and $[\eta]_G$ are those of native collagen and completely-denatured gelatin respectively. The effect of the temperature on the intrinsic viscosity appeared from 30°C and vanished above 40°C as is shown in Fig. 1. The denaturation temperature can probably be identified as 38°C from those experimental results too.

Flow Birefringence. By assuming that a molecule of collagen is a thin rod, the rotational diffusion coefficient at a certain velocity gradient, (Θ_G) can be determined using the table of Scheraga *et al.*[12] Moreover, the rotational diffusion coefficient at the zero velocity gradient ($\Theta_G=0$) can be obtained from the tangent at the zero velocity gradient in a plot of the extinction angle *versus* the velocity gradient by the Peterlin-Stuart equation.[13] These values at various velocity gradients are shown in Table 1.

Table 1. The rotational diffusion coefficient and computed length of collagen molecule as a function of velocity gradient at concentration of 0.0544% at 25°C

Velocity gradient sec^{-1}	Rotational diffusion coefficient sec^{-1}	Length Å
0	(824)	(3000)
850	833	2990
1220	870	2940
1710	854	2960
1980	840	2980
2210	916	2890
2460	944	2860
2700	909	2900
2930	961	2850
3940	980	2830
4160	909	2900

11) C. Cohen, *J. Biophys. Biochem. Cytol.*, **1**, 203 (1955).
12) H. A. Scheraga, J. T. Edsall and J. O. Gadd, Jr., *J. Chem. Phys.*, **19**, 1101 (1951).
13) A. Peterlin and H. A. Stuart, *Z. Physik*, **112**, 129 (1929).

The middle value was 909 sec^{-1}, from which the value at 20°C in water was estimated to be 795 sec^{-1}.

Sedimentation. The sedimentation pattern of collagen was very sharp. The sedimentation coefficient of collagen in water at 20°C ($s_{20,w}$) was calculated at several concentrations. The plot of its reciprocal value *versus* the concentration is shown in Fig. 4. The following relation is obeyed:

$$\frac{1}{s_{20,w}}=\frac{1}{s^\circ_{20,w}}(1+k_s c)$$

where k_s is a constant and $s^\circ_{20,w}$ the sedimentation coefficient at an infinite dilution. From the figure the values of k_s and $s^\circ_{20,w}$ were found to be 2.4 dl/g and 3.0 S respectively. The value of the sedimentation constant thus obtained lies within the range of the values of the sedimentation constants of the collagen of other species (2.8—3.5 S).[5]

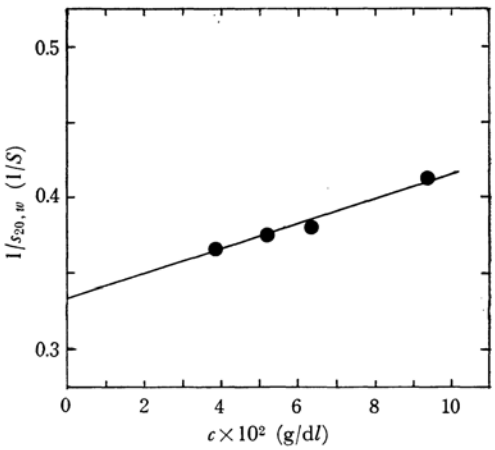

Fig. 4. The concentration dependence of sedimentation coefficient of collagen at 20°C in water.

It is known that the fiber of collagen shrinks above a certain temperature, *i. e.*, the shrinkage temperature.[5] That of chicken leg tendon was found to be 65°C. This is almost identical with the value reported by Leach (64°C).[8]

Discussion

The correlation between the denaturation temperature and the estimated frequencies of collagen triplets devoid of pyrrolidine residues in the various soluble collagens has been discussed by Josse and Harrington.[14] For the collagen of chicken leg tendon, this correlation holds when the amino acid composition reported by Leach[8] is adopted.

The denaturation temperatures of different collagens under the same experimental conditions

14) J. Josse and W. F. Harrington, *J. Mol. Biol.*, **9**, 269 (1964).

seem to be functions of the environmental temperature of the animal from which the collagen was extracted. For example, the denaturation temperature of the collagen from the skin[15] and the swim bladder[7,15] of cod, which lives in the cold sea, was 13—16°C, and that of the ichthyocol of the swim bladder[6] of fresh-water carp was 29°C, while that of rat skin[7] was 36°C and that of carf skin,[16] 36°C. The denaturation temperature of chicken tendon was found to be 38°C. This higher denaturation temperature may reflect the higher body temperature of fowls.

The molecule of the collagen was assumed to be prolate ellipsoid in shape, because it has generally been known to be a rod as a result of light-scattering experiments[6,16] and electron microscopy. The following equation can be derived from Simha's Equation,[17] if p is 150^{-1} -200^{-1}:

$$\frac{M[\eta]}{NV_e} = \frac{4.22(1/p)^2}{15[\{\ln(2/p) - 1/2\}} \tag{1}$$

where M is the molecular weight; V_e, the effective volume[18]; p, an axial ratio of the prolate ellipsoid; N, the Avogadro number, and $[\eta]$, the intrinsic viscosity. In this case, p equals b/a, where b is the equatorial radius of revolution and a is the length of the semi-axis.

The effective volume is written by using Perrin's Equation[19] in the following way:

$$V_e = \frac{4\pi ab^2}{3} = \frac{kTp^2\{\ln(2/p) - 1/2\}}{2\eta\Theta} \tag{2}$$

where k is the Boltzmann constant; T, the absolute temperature; η the viscosity of the solvent, and Θ, the rotational diffusion coefficient. From the above equations, we get:

$$M = \frac{2.11kTN}{15\eta\Theta[\eta]} \tag{3}$$

As is shown in Eq. (3) the molecular weight can be determined without a knowledge of the effective volume if the rotational diffusion coefficient and intrinsic viscosity are known. The molecular weight of the collagen was determined by using this equation to be 34×10^4.

The effective volume is given in terms of the partial specific volume, $\bar{v}$, as follows:

$$V_e = \frac{M}{N}\left(\bar{v} + \frac{w}{\rho_0}\right) \tag{4}$$

where w is the number of grams of solvent bound per gram of dry protein, and where ρ_0 is the density of the solvent. From Eqs. (2) and (4) and the sedimentation constant, $s^\circ_{20,w}$, related to an axial ratio, the following equation can be derived:

$$\frac{5.83\times10^3 s^3\Theta^2(\bar{v}+w/\rho_0)^3\eta^5\pi^2}{k^2T^2(1-\bar{v}\rho_0)^3} = (9/4)p^6\{2\ln(2/p)-1\}^2\{\ln(2/p)\}^3 \tag{5}$$

Assuming several different values of bound water (w=0, 0.1, 0.2, 0.25, and 0.3), the values of the partial specific volume and the axial ratio were determined by seeking for a value of the partial specific volume so as to have the same value of the axial ratio in Eq. (5) as in Eq. (1) (Table 2). By substituting these obtained values of the partial specific volume and the axial ratio into Eq. (5) or (1), the molecular weight was calculated to be as shown in Table 2. In Table 2, both the partial specific volume and the molecular weight are shown to be independent of the quantity of hydration. However, the axial ratio depends on the hydration. The molecular weight thus calculated was almost identical with that obtained from Eq. (3) which can be compared with the value of 345000 for the ichthyocol[6] obtained by the light-scattering method. The molecular weight determination by the light-scattering measurement can be made without a knowledge of the partial specific volume. The axial length can be obtained by Perrin's Equation.[19] When the value of p^{-1} changes from 148 to 179, the value of $[-1+2\ln(2/p)]^{1/3}$ changes from 2.18 to 2.21. The difference between those values is less than 2%. The molecular lengths calculated by Perrin's Equation[19] are shown in Table 1, assuming p^{-1} as 179. These values are not very different from those obtained by assuming p^{-1} as 148. In the flow-birefringence experiment with a polydisperse system, the longer molecules with smaller rotational diffusion coefficients easily orient at smaller velocity gradients than the shorter molecules. The greater the velocity gradient, the shorter molecules with greater rotational diffusion coefficients can begin to orient themselves. In Table 1 we can see a slight indication of the polydispersity of this chicken collagen.

Table 2. Partial specific volume, axial ratio and molecular weight obtained from Eqs. (1) and (5) when various quantities of hydration were assumed

w (grams per gram of collagen)	$\bar{v}$ ml/g	$1/p$	$M_w\times10^4$
0	0.747	179	33—34
0.10	0.750	166	34—35
0.20	0.745	157	34
0.25	0.748	152	35
0.30	0.744	148	34

15) E. Gordon Young and J. W. Lorimer, *Acrh. Biochem. Biophys.*, **92**, 183 (1961).
16) P. Doty and T. Nishihara, "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.), Pergamon, London (1958), p. 92.
17) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).
18) H. A. Scheraga, "Protein Structure," Academic Press, New York (1961).
19) F. Perrin, *J. phys. radium*, [7], **5**, 497 (1934).

Table 3. Molecular weight, 2a and 2b obtained from each couple of intrinsic viscosity, rotational diffusion coefficient and sedimentation constant
(assumed to be 0.70 ml/g as partial specific volume and NV_e/M)

	$[\eta]$	$[\eta]-\Theta_{20,w}$	$\Theta_{20,w}-s^\circ_{20,w}$	$s^\circ_{20,w}-[\eta]$
M		32.1×10^4	28.4×10^4	26.4×10^4
2a (Å)		2900	2980	2720
2b (Å)		15.7	14.5	14.7
$1/p$	185		205	

The molecules, however, are much more homogeneous than those of the collagen from rat tail tendon.[20] The chicken collagen might be compared with that of the ichthyocol of carp swim bladder.[6] The collagen is slightly soluble in water, and its solution is too viscous even in a highly diluted solution. For these reasons the determination of its partial specific volume is so difficult that it is usually assumed to be identical with that of gelatin, which can be rather easily determined. When the partial specific volume is assumed to be 0.70 ml/g, that of gelatin,[5,6,21] the molecular weight and the molecular sizes calculated from different sets of data do not coincide with each other, as is shown in Table 3. As has been mentioned above, the soluble chicken collagen is nearly monodisperse. Therefore, the molecular weights obtained from each set of the data are expected to be identical. If the effective volumes should be identical in all three different hydrodynamic methods, a value larger than 0.70 ml/g is more plausible as the partial specific volume of this collagen.

In conclusion, the collagen of chicken leg tendon is 2900—3000 Å in length and 12—16 Å in diameter, and has a molecular weight of 34×10^4.

The authors wish to express their hearty thanks to Mrs. A. Akaboshi for her technical help in the sedimentation experiment.

20) H. Noda, *Nippon Kagaku Zassi (J. Chem. Soc. Japan, Pure Chem. Sect.)*, **79**, 767 (1958).

21) P. N. Gallop, *Arch. Biochem. Biophys.*, **54**, 501 (1955).