Memoirs

of the

Institute for Protein Research

Osaka University

EDITORS

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VOL. 14

INSTITUTE FOR PROTEIN RESEARCH
OSAKA UNIVERSITY

OSAKA JAPAN

1972

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(Abstract)

(Seminars)

J. Biochem., **69**, 43–52 (1971)

Sedimentation Analysis of Soluble Collagen and Its Subunits of Chicken Leg Tendon

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Received for publication, April 24, 1970

The description of some physico-chemical properties of soluble collagen extracted from chicken leg tendons in solution have been provided. The sedimentation equilibrium experiments were carried out. The molecular weight of soluble collagen was found to be 231,000. When soluble collagen was treated with guanidine hydrochloride to destroy collagen structure effectively, its molecular weight was reduced to one-third, 76,000. Furthermore, the molecular weight of heat denatured collagen was found to be 18,500, which is a value of one-twelfth of that of native collagen in an aqueous solution.

For the soluble collagen the concentration dependence of apparent molecular weights, sedimentation coefficients, and viscosities, suggest that the collagen molecule has a rod shape, because the second virial coefficients are larger than that of globular protein molecules of the same molecular weights.

Data of the heat denatured collagen require the consideration of an association mechanism. It has become possible to calculate the dimerization constant and to assign the sedimentation coefficients for dimer and monomer.

The length of the collagen molecule which was obtained with hydrodynamic investigations agreed to our results of electron microscopy.

Collagen is contained in connective tissues of animals and generally said to amount to about 90% of the proteins in animals. Corresponding to this fact, many workers have investigated the properties of collagen for a long time. However, the investigation of collagen at the molecular level has been difficult, because collagen dose not have so-called physiological activities and because most collagen fibrils are insoluble.

There have been many investigations of the molecular weight of collagen. The most famous is that of Boedtker and Doty (*1*), who used soluble collagen extracted from carp swim bladder by a means developed by Gallop (*2*). The collagenous tissue was washed with an aqueous sodium acetate solution and soaked in a citrate buffer solution at pH 4.3. The extract was dialyzed against an aqueous disodium hydrogen phosphate solution and the precipitate which appeared in the dialyzing bag was collected. They measured the molecular weight of this collagen by light scattering and reported the weight average molecular weight of 345,000±30,000. However, the number average molecular weight, measured with osmotic

pressure, was reported as 310,000±50,000, and that calculated by combining Perrin's and Simha's eq. (*3*) with intrinsic viscosity and sedimentation coefficient as 250,000.

In contrast to those, Noda reported that the molecular weight of rat tail tendon collagen was 510,000 obtained from intrinsic viscosity and sedimentation coefficient (*4*).

As a result of many other investigations of the molecular weight of collagen, extracted from various sources, it has been generally accepted that the molecular weight of collagen is about 300,000. As an example showing the smaller values of molecular weight, Stainsby reported that the molecular weight of calf skin collagen, extracted with a dilute alkali solution, was 250,000–270,000 (*5*).

These large discrepancies in the molecular weight of collagen, probably being due to the procedures of extraction and measurement, indicate difficulty in determining the molecular weight of collagen.

The structure of the collagen molecule is also problematic. Ramachandran analyzed its structure by X-ray diffraction (*6*) and proposed that the collagen molecule consisted of three peptide chains twisted together along a common axis of rotation.

Doty and Nishihara supported this view of a three stranded structure through their investigation of the following phenomena (*7*). After heating calf skin collagen at 40°C in a citrate buffer solution, they made a sedimentation analysis and found two peaks on a sedimentation pattern whose sedimentation coefficients were respectively 2.85S and 5.4S. After the collagen solution to which was added 0.13 M sodium chloride and 1.2 M potassium thiocyanate, was kept at pH 12 overnight, the faster peak disappeared.

These lead to the conclusion that collagen might separate into a double stranded chain and a single chain, and then separate into three single chains.

Gallop found that the separated chains with addition of hydroxylamine or hydrazine, were degraded into smaller subunits, of which molecular weights were 25,000–30,000 (*8*).

Although there have been many investigations of collagen and its subunits, there remain many obscure properties to be solved because the collagen solution is so unstable that its components are apt to associate from each other.

The present authors have recently found a novel method to extract collagen from chicken leg tendons. The collagen thus obtained is more stable and is suitable for the elucidation of physico-chemical properties of collagen more definitely. In the present work, some results on sedimentation analysis and other related properties of this collagen in various conditions will be described and a subunit structure of the collagen molecule is proposed.

MATERIALS

Achilles tendons were separated from chicken legs and stripped of their meat and fat. They were cut into about 1 cm in length and soaked in 10% aqueous sodium chloride solution for two hours. Then they were washed with distilled water till they became free from sodium chloride. Next they were dipped in distilled acetone. To wash acetone soluble materials away, acetone was removed several times. After being soaked in acetone for a week with several exchanges of acetone, the minced tendons were soaked again in a 10% aqueous sodium chloride solution while renewing the solution several times for two days. After being washed with distilled water to be free from sodium chloride, they were soaked in distilled water, to which hydrochloric acid was added. The suspension was brought to pH 3.0 and kept at 4°C. To keep the suspension at pH 3.0, occasional addition of dilute hydrochloric acid was necessary during the course of time. The minced tendons became to swell out while being kept for several days under these conditions. After one week, the extract was strained through cotton gauze and then centrifuged at 77,000×*g* for one hour so that the precipitates which passed through the cotton guaze were removed. The supernatant solution was dialyzed against distilled water with Visking membrane in order to remove hydrochloric acid. As the solution became neutral, white collagen gel appeared in the dialyzing bag, which was collected and dried

by lyophilization. Then the dried materials were stored in a desiccator. To make up collagen solutions, the dried sample was dissolved in an acetate buffer solution at pH 4 (0.01 M sodium acetate+acetic acid with 0.1 M sodium chloride). Because there were small amounts of insoluble collagen, the solution was centrifuged at $60,000\times g$ and dialyzed against the above stated solvent.

In order to make a collagen solution with guanidine hydrochloride, dried sample was dissolved in 6 M aqueous guanidine hydrochloride solution in a cold room. Guanidine hydrochloride was recrystallized from methanol. In this case, a small amount of insoluble parts were left, which was removed by centrfugation.

Solution of heat denatured collagen was prepared in the following way. Dried sample was dissolved in an acetate buffer solution as stated above. Since less than 0.3% of the dried sample was soluble, the remainder formed a suspension after a while. Then the suspension was heated for twenty minutes in a water bath at 80°C. Concentrations were calculated from nitrogen analysis except for the guanidine hydrochloride solution.

Nitrogen analyses were carried out by micro-Kjeldahl method. With the result of the amino acid analysis, collagen was assumed to contain 15.2% nitrogen.

EXPERIMENTAL METHODS

1) *Electron Microscopy*–Electron microscopy was carried out with JEM 7 (manufactured by the Japan Electric Optics Laboratory Co. Ltd.). The suspension of the sample was dropped on Formval film which was spread on copper grids. Platinum-palladium alloy was used for shadow casting.

2) *Ultracentrifuge*–All of ultracentrifugal measurements were carried out with a Beckman-Spinco Ultracentrifuge Model E. Molecular weights were determined by the sedimentation equilibrium method with double sector cells which were made of Filled Epon. The apparent molecular weight is given by the following equation,

$$M_{app}=\frac{2RT}{(1-\bar{v}\rho)\omega^2}\,\frac{\ln C_b-\ln C_a}{b^2-a^2} \qquad (1)$$

where R is the gas constant, T is absolute temperature, $\bar{v}$ is partial specific volume, ω is the rotor speed, a and b are the distance to meniscus and to bottom of the liquid column from the center of the rotor, respectively, C_a and C_b are the concentration of protein at the meniscus of the centrifugal cell and at the bottom. Native collagen solution was centrifuged at 5,227 rpm at 25°C, for 48 hr by using an An-J rotor. Collagen in 6 M guanidine hydrochloride solution was centrifuged with 12,590 rpm, at 20°C, for 24 hr and heat denatured collagen solution was centrifuged with 12,590 rpm at 30°C, for 24 hr by using an An-E rotor.

For heat denatured collagen solution M_{app} is an apparent molecular weight defined by the equation,

$$M_{app}=\frac{2RT}{(1-\bar{v}\rho)\omega^2}\,\frac{d\ln C}{dr^2} \qquad (2)$$

where C is the solute concentration. The tangent to the experimentally determined curve for $\ln C$ against r^2 was evaluated at the midpoint between the meniscus and bottom within the cell. Sedimentation coefficients was determined at 47,590 rpm by using a Filled Epon double sector cell.

3) *Determination of Concentration Dependence of the Refractive Index*–The refractive indices were measured with a Brice Phoenix Differential Refractometer. The constant of the instrument was calculated with saturated aqueous potassium chloride solution by using the value in "International Critical Table." Special grade potassium chloride was purchased from Nakarai Chem. Ltd.

4) *Concentration Determination with Interference Optics*–In centrifugation, the concentration is given by the following equation using a synthetic boundary cell.

$$J=\frac{\hat{a}}{\lambda}\left(\frac{dn}{dC}\right)C \qquad (3)$$

where $\left(\frac{dn}{dC}\right)$ is the specific refractive index increment, λ is the wavelength, 5.46×10^{-5} cm, $\hat{a}$ is the light path in the cell, 1.2 cm, C is the concentration of solute in g/dl, J is the fringe displacement. The concentration determinations were calculated with this method

and nitrogen analyses as stated in the preceding section. Concentrations determined by the two different methods were in good agreement with each other. Concentration of collagen in 6 M aqueous guanidine hydrochloride solution was calculated only by Eq. (3).

5) *Partial Specific Volume*—Partial specific volumes were determined with pycnometric measurements. The volumes of the used pycnometers were about 50 ml for the collagen solution and about 25 ml for the heat denatured collagen solution. The accuracy of temperature control of the water bath was ±0.02°C and the volumes of the pycnometers were calibrated with the density of water stated in "International Critical Table."

6) *Viscosity*—Viscosity determinations were carried out with a Ubbelohde-type viscometer. The flow time of the viscometer was 237 sec. Temperature was 25±0.01°C for the collagen solution and 35.00±0.01°C for the heat denatured collagen solution.

RESULTS

1) *Electron Microscopy*—Collagen was dissolved in an acetate buffer solution (0.1 M NaCl +0.01 M $CH_3COONa+CH_3COOH$ at pH 4.0). After the solution was kept at room temperature for about two weeks, white precipitate occurred from the solution. The electron micrograph is shown in Fig. 1, which clearly indicates the characteristics of collagen fibrils. The repeating period of the band is about 700Å. Such 700Å long bands have been found in intact native fibrils and precipitates salted out with 0.1 M NaCl (*9*).

2) *Amino Acid Analysis*—Collagen in a

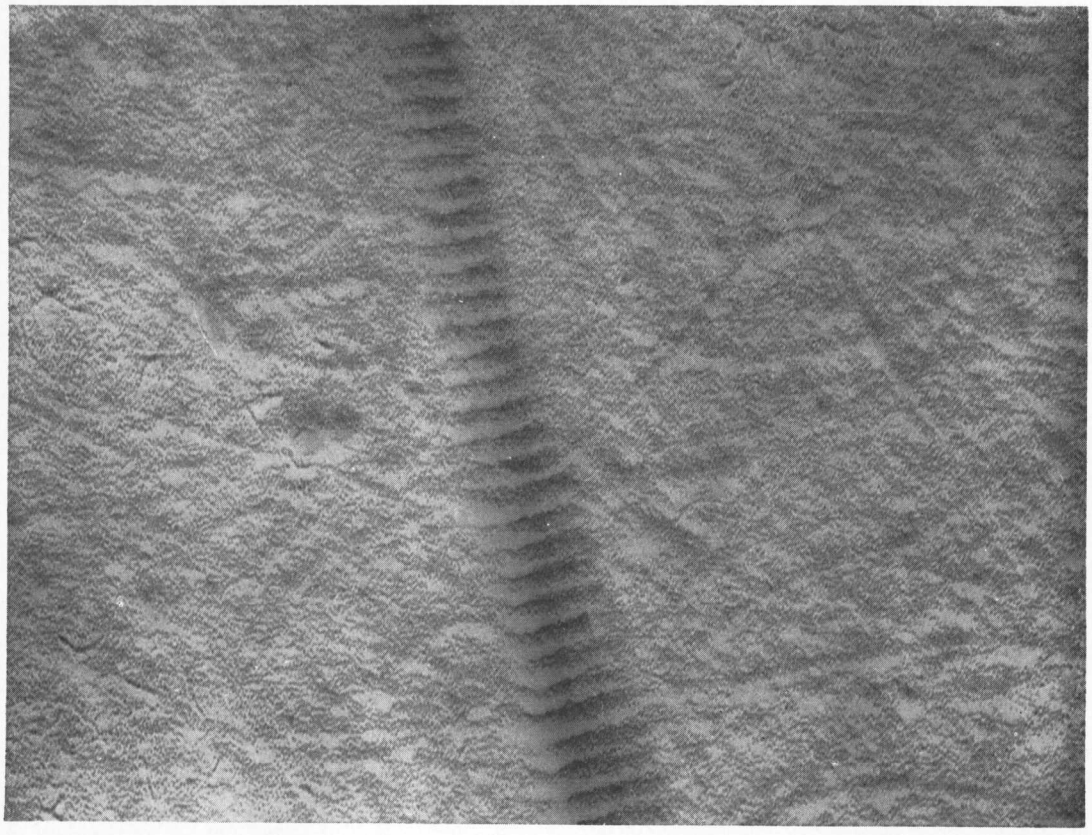

Fig. 1. Electron micrograph of the collagen fibrils of chicken leg tendon. The sample was shadow cast with platinum-palladium alloy after being dried. Magnification ×42,000

6 N hydrochloric acid solution was hydrolyzed for twenty hours at 110°C. The digested products were analyzed with a Beckman-Spinco Model MS amino acid analyzer. At two different temperatures of 50°C and 30°C, analyses were carried out in order to obtain a complete result. The amino acid composition of collagen is listed in Table I.

TABLE I. Amino acid composition of soluble collagen of chicken leg tendon.

	Amino acid residue weight per 100 g weight	Residue number per mole (M.W.=231,000)
Lis	3.18	57.0
His	0.78	13.1
Arg	8.42	124.1
Hylys	2.10	37.6
Asp	5.78	115.5
Thr	2.09	47.5
Ser	2.45	68.1
Glu	10.76	191.8
Pro	13.00	308.1
Gly	20.00	805.6
Ala	8.93	289.1
Val	2.15	49.8
Met	1.09	19.1
Ileu	1.52	31.0
Leu	1.71	34.9
Tyr	0.76	10.6
Phe	2.47	38.6
Hypro	12.86	304.7
Total	100.05	2546.2

3) *Partial Specific Volume*—The partial specific volume of collagen was deduced to be 0.69_5 ml/g, and that of heat denatured collagen was to be 0.68 ml/g.

4) *Molecular Weight*

Collagen—Apparent weight average molecular weights of native collagen were determined by sedimentation equilibrium at six collagen concentrations. The relationship between the reciprocal values of these apparent molecular weights and collagen concentration, $\bar{C}$, is shown in Fig. 2. The extrapolation of concentration to infinite dilution is given by the following equation.

$$1/M_{app}=1/M_w+2A\bar{C} \qquad (4)$$

where M_{app} is the apparent molecular weight at a given collagen concentration, M_w is the weight average molecular weight, and $\bar{C}$ denotes an average concentration defined by

$$\bar{C}=(C_a+C_b)/2 \qquad (5)$$

The extrapolated values of $1/M_{app}$ for collagen was 4.30×10^{-6} so that the corrected molecular weight was 231,000 and the second virial coefficient A was 3.8×10^{-4} cgsu.

Collagen in 6 M Aqueous Guanidine Hydrochloride Solution—Because of the difficulty is experimental determination the partial specific volume of collagen was assumed to be 0.68 ml/g in 6 M aqueous guanidine hydrochloride solution. The relationship between $1/M_{app}$ and concentration, $\bar{C}$, is shown in Fig. 3. The extrapolated value for collagen in 6 M guanidine hydrochloride solution was 1.33×10^{-5} so that the corrected molecular weight was 75,000.

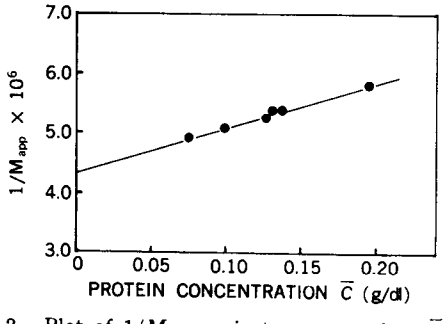

Fig. 2. Plot of $1/M_{app}$ against concentration, $\bar{C}$, for soluble collagen in acetate buffer (pH 4.0, ionic strength approximately 0.1) at 25°C.

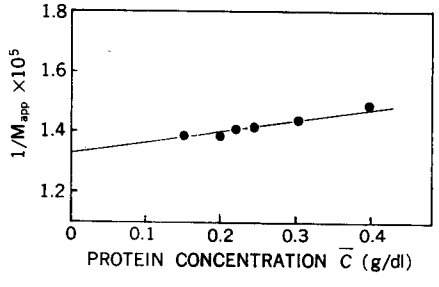

Fig. 3. Plot of $1/M_{app}$ against concentration for soluble collagen in 6 M guanidine hydrochloride at 20°C.

The second virial coefficient was 2.3×10^{-4} cgsu which was a half of that of collagen.

Heat Denatured Collagen—Apparent molecular weight of collagen denatured by heat at 80°C for 20 min was determined with a centrifuge rotor which was kept at 30°C. The relationship between M_{app} and concentration, C, is shown in Fig. 4. The apparent molecular weight increases slightly with the increase of concentration in dilute concentration region so that the presence of association of the subunits of collagen have to be considered in our experimental condition. The corrected molecular weight extrapolated to zero concentration was found to be 18,500. The summarized data are listed in Table II.

5) *Sedimentation Coefficient*—The relation between reciprocal values of sedimentation coefficients of collagen, $s_{25,w}$, and concentration is shown in Fig. 5. The following equation gave 3.4S as $s^\circ_{25,w}$ and 4.3 dl/g as k_s

$$1/s_{t,w}=1/s^\circ_{t,w}(1+k_sC) \qquad (6)$$

where $s_{t,w}$ is the sedimentation coefficient at a given concentration of C in water, $s^\circ_{t,w}$ is the corrected sedimentation coefficient at $C=0$ in water, and k_s is a constant. In the same way, for collagen in 6 M guanidine hydrochloride solution $s^\circ_{20,w}$ was calculated to be 1.5_6 S and to be 10.51 dl/g as k_s. The sedimentation coefficient of the heat denatured collagen was about 2 S. In this case, an association-dissociation system had to be taken into consideration. The analysis of this system in detail is in the following section. The relationship between the values of the sedimentation coefficient in these two system is shown in Figs. 6 and 7.

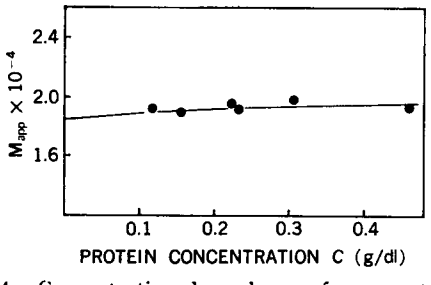

Fig. 4. Concentration dependence of apparent molecular weight (M_{app}) of heat denatured collagen (incubated for 20 min at 80°C) in acetate buffer at 30°C. Solid curve: calculated by Eq. (8).

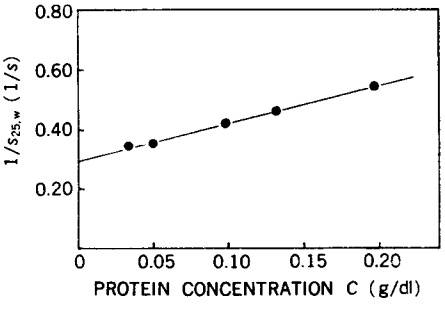

Fig. 5. Plot of $1/s_{25,w}$ against concentration for soluble collagen in acetate buffer (pH 4.0, ionic strength approximately 0.1).

TABLE II. Summary of molecular parameters obtained for the soluble collagon of chicken leg tendon.

	Native collagen	6 M Guanidine-HCl	Head denatured collagen
M_w	231,000	76,000	18,500
$s^\circ_{20,w}$ (S)	2.95	1.56	Monomer 1.75 Dimer 2.2
k_s (dl/g)	4.3	0.51	0.1
A_2 (cgsu)	3.8×10^{-4}	2.3×10^{-4}	2.0×10^{-4}
$\bar{v}$ (ml/g)	0.69_5	0.68[1]	0.68
K_2' (dl/g)	——	——	0.29
$[\eta]$ (dl/g)	13.5 (at 25°C)	——	0.346 (at 30°C)
$\frac{dn}{dC}$ (dl/g)	1.71×10^{-1}	1.12×10^{-1}	1.87×10^{-1}

[1] Assumed to be indentical with that of heat denatured collagen.

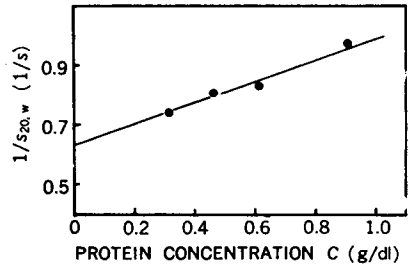

Fig. 6. Plot of $1/s_{20,w}$ against concentration for soluble collagen in 6 M guanidine hydrochloride.

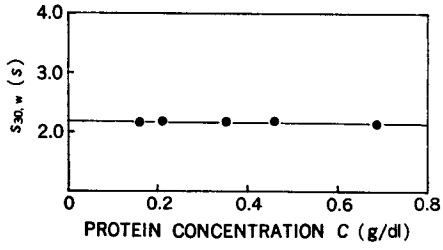

Fig. 7. Plot of sedimentation coefficient, $s_{30,w}$, against protein concentration for heat denatured collagen. Solid curve: calculated by Eq. (9).

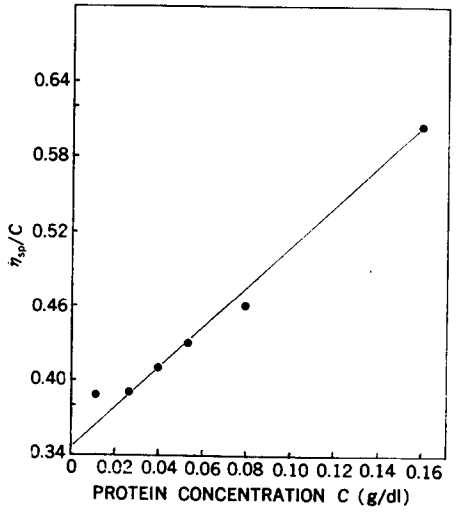

Fig. 8. Plot of reduced viscosity (η_{sp}/C) against protein concentration for soluble collagen in 0.1 M acetate buffer of pH 4.0 at 25°C.

6) *Viscosity*—In Fig. 8, the values of the reduced viscosity of the collagen solution are plotted *versus* concentration. Intrinsic viscosity is given by the following equation.

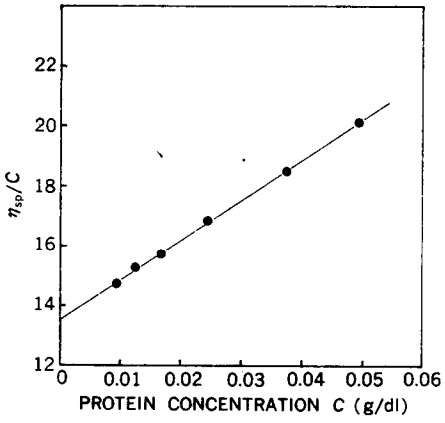

Fig. 9. Plot of reduced viscosity (η_{sp}/C) against protein concentration for heat denatured collagen (incubated for 20 min at 80°C) in 0.1 M acetate buffer of pH 4.0 at 35°C.

$$[\eta]=\lim_{C\to 0}\frac{\eta_{sp}}{C} \qquad (7)$$

where $[\eta]$ is intrinsic viscosity, η_{sp} is specific viscosity and C is concentration. The intrinsic viscosity of collagen was 13.5 dl/g which was unusually larger than that of other common protein. The intrinsic viscosity of the heat denatured collagen was 0.35 dl/g. The relationship between the reduced viscosity of the heat denatured collagen and the concentration is shown in Fig. 9. The relation was linear so that the association-dissociation system was not detectable in the range of collagen concentration where the measurements were carried out. In Table II the molecular parameters of collagen are tabulated.

DISCUSSION

The molecular weight of collagen was calculated to be 231,000, which was less than the generally accepted 300,000 or thereabout. The reason why there is a difference between them is not yet known. It might depend on the manner of purification of collagen. Doty and his coworkers took polypeptide chains which were named parent gelatin, by heating a collagen solution at 40°C for 10 min. Gallop and his coworkers studied, in detail, this so-called parent gelatin by various methods including

sedimentation equilibrium. The parent gelatin has a broad molecular weight distribution.

In our investigation, the parent gelatin of chicken tendon collagen by the same procedure as that of Gallop *et al.*, was measured by a sedimentation equilibrium method at 25°C, also the same as Gallop used. When the logarithm of fringe scale, J, was plotted *versus* the square of distance from the center of rotor, r^2, the curve was concave upward (not shown in figure). The molecular weights were estimated from the derivatives of this curve. It gave about 100,000 at the meniscus and amounted to 500,000 at the bottom.

We will discuss below how this discrepancy arose between Gallop *et al.* and ours in homogeneity as well as in molecular weight of parent gelatin. In the collagen solution which has been heated at 40°C for 10 min, there will be not only polypeptides which have formed by unfolding and splitting of the native collagen but also there will be formed smaller units of the polypeptides which are fragments of broken pieces. Moreover only by heating at 40°C for 10 min, the structure of collagen has not been completely destroyed, and it should recover partially at 25°C, the temperature at which Doty and Gallop measured their molecular weights. Considering the situation which those indicate, it would be almost impossible to determine the molecular weight of parent gelatin itself separately from the other species. We have investigated, in detail, "unfolding-refolding process of collagen molecule" by circular dichroism spectroscopy. The results of that analysis are in good agreement with above discussion (*10*).

In our investigation, guanidine hydrochloride which is supposed to destroy effectively protein structure, was used, and the molecular weight of collagen in 6 M aqueous guanidine hydrochloride solution was found to be 75,000 which was a good approximate value of one-third of the molecular weight of collagen in aqueous solution, 231,000. It should be emphasized that molecular weights obtained here were determined on the collagen samples, either native or guanidine hydrochloride denatured, both by means of the sedimentation equilibrium.

The molecular weight of heat denatured collagen was found to be 18,500 which was a rather better approximate value of one-twelfth of the molecular weight of collagen in an aqueous solution, 231,000. This agrees well with the hypothesis which is taken from the following results of electron microscopy and chemical studies. The polypeptides of collagen were degraded into subunits of which molecular weights were 25,000–30,000 with nucleophilic reagents such as hydroxylamine and hydrazine (*11*). The cleaved points are said to be ester-like bonds. The length of the collagen molecule is assumed to be about 2,700 Å. There are characteristic periodical bands in a collagen fibril. The length between these bands is about 700 Å and its value is one-forth of that of collagen molecule might have special periodical characteristics. If these characteristics would correspond to the position of ester-like bonds on a collagen molecule which exists on every one of three chains, and which would make the peptide chain into four subunits with heating, the whole of collagen molecule would be degraded into twelve subunits. It would be reasonable to assume that such ester-like bonds would be cleaved before the polypeptide bonds were cleaved by heating at 80°C for 20 min. In our investigation, the molecular weight determination of the heat denatured collagen was carried out at 30°C. If it were possible it would be better to perform the measurements at a higher temperature.

Because there is an association-dissociation equilibrium at 30°C, the following investigation was carried out. This system is assumed as an association-dissociation system of a monomer, of which the molecular weight is 18,500, and a dimer. If the equilibrium constant for the association of monomers into a dimer is expressed by K_2', this system can be described by the following equation (*12*).

$$\frac{M_{app}}{2M_1 - M_{app} - 4AM_1M_{app}} = 1 + 4K_2'C \qquad (8)$$

where M_1 is the molecular weight of the monomer, 18,500, and A is the second virial coefficient which is assumed to be identical for both monomer and dimer. Now A is as-

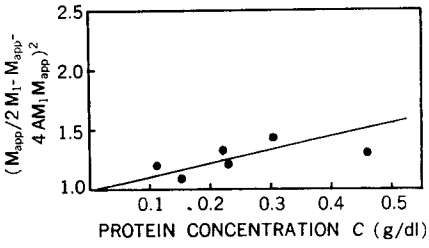

Fig. 10. $[M_{app}/(2M_1-M_{app}-4A\ M_1\ M_{app})]^2$ plotted against C for heat denatured collagen.

sumed to be 2×10^{-4} dl/g. Apparent molecular weights were given by the sedimentation equilibrium measurement at six different concentrations for heat denatured collagen. The quantity of $[M_{app}/(2M_1-M_{app}-4AM_1M_{app})]^2$ is plotted against concentration in Fig. 10. From the inclination, K_2' was found to be 0.30 dl/g. Conversely, M_{app} was calculated by substituting this K_2' value for Eq. (8). The calculated M_{app} values have been drawn as a curve in Fig. 4 which passes the points of observed M_{app}. Extrapolation of this curve which is taken with calculated values to zero concentration yields 18,500 for M_1.

If the sedimentation coefficient of the mixed solution of the monomer and the dimer is expressed by s, it is given by the following equation (13).

$$s=(s_1+s_2K_2'C)/(1+K_2'C) \qquad (9)$$

where s_1 and s_2 are sedimentation coefficients of the monomer and the dimer, respectively. The concentration dependence of the sedimentation coefficient is described by the following equation (14),

$$s_1=s_1^0/(1+k_sC) \qquad (10)$$

$$s_2=s_2^0/(1+k_sC) \qquad (11)$$

where s_1^0 and s_2^0 are sedimentation coefficients at the infinite dilution, k_s is a constant. The coefficient k_s is assumed to be identical for both monomer and dimer. Substituting Eqs. (10) and (11) into Eq. (9),

$$s=\frac{s_1^0-s_2^0}{(1+K_2'C)(1+k_sC)}+\frac{s_2^0}{1+k_sC} \qquad (12)$$

The correlation between the reciprocal values of s and C is linear in the region where C is large enough. Extrapolation of this linear relation to zero concentration, gives s_2^0 and the inclination gives k_s. Substituting the observed sedimentation coefficient into (12), s_2 is found to be 2.8 S and k_s to be 0.1 dl/g. Eq. (12) may be rewritten as

$$s_2^0-s(1+k_sC)=\frac{s_2^0-s_1^0}{1+K_2'C} \qquad (13)$$

Plotting $1/(1+K_s'C)$ with $s_2^0-s(1+k_sC)$, k_s and K_2' are as previously expected. $(s_2^0-s_1^0)$ is taken from the inclination and s_1 is calculated to be 2.2 S. Using those values for s_1 and s_2, the curve in Fig. 7 can be obtained for s at a given concentrations, C. It is seen that the calculated curve reproduces the experimental values very well.

It can be concluded that a collagen molecule extracted from chicken leg tendon by hydrochloric acid consists of three polypeptide chains. Each polypeptide chain has the molecular weight, 75,000, which is approximately one-third of the native collagen. Collagen molecule unfolds and dissociates into those three polypeptide chains, previously called parent gelatin, when exposed to 6 M guanidine hydrochloride. The parent gelatin further degrades into four subunits upon heating at 80°C. However, those subunits are in mutual equilibrium with each other, and the fraction of units in associated forms increases with increasing concentration. The monomeric form of the subunit has the molecular weight of 18,500 and sedimentation coefficient of 2.2 S, while its dimeric form has the sedimentation coefficient, 2.8 S.

The shape of the native collagen molecule in an aqueous solution was investigated by hydrodynamic procedures. In general, the frictional ratio f/f_0 of a hydrated protein molecule in solution is given by

$$f/f_0=1.19\times10^{-15}\frac{M^{2/3}(1-\bar{v}\rho)}{s^\circ_{20,w}\bar{v}^{1/3}} \qquad (14)$$

where M is the molecular weight of protein, $\bar{v}$ is its partial specific volume, s is the sedimentation coefficient, and ρ is the density of solution (*15*). Substituting the numerical data: M is 231,000; $\bar{v}$ is 0.695 ml/g; ρ is 1.001 g/ml; s is 2.95×10^{-13} sec^{-1}, then the frictional

ratio for native collagen is calculated to be 5.09. For the prolate ellipsoids, Perrin gave the following equation about the correlation between f/f_0 and axial ratio (*16*).

$$f/f_0 = \frac{(1-b^2/a^2)^{1/2}}{(b/a)^{2/3} \ln \dfrac{1+(1-b^2/a^2)^{1/2}}{b/a}} \qquad (15)$$

where a and b are length of the major semi-axis and minor semi-axis, respectively. If the shape of collagen is assumed to be a prolate ellipsoid, a/b is calculated to be 159 using the value f/f_0. If the shape of collagen is assumed to be a rod, whose volume is considered to be equivalent to that of a prolate ellipsoid the following equation is given (*17*).

$$\frac{a}{b} = \left(\frac{2}{3}\right)^{1/2} \frac{L}{d} \qquad (16)$$

where L is the length of the rod and d is the diameter. Substituting the value of a/b taken above, and assuming d to be 14.5 Å, L is calculated to be 2,800 Å. This length of the rod agrees with the result we have found from electron microscopy.

The authors wish to thank Prof. T. Isemura for his encouragement and Dr. N. Yamamoto for valuable suggestion to the preparation of soluble collagen. They wish to express their thanks to Dr. R. Nagai for technical assistance in electron microscopy.

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BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN, VOL. 44, 1416—1417 (1971)

Asymmetric Hydrogenation of C=O Double Bond with Modified Raney Nickel Catalyst. XVI

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(Received June 8, 1970)

In the course of the investigation of the asymmetrically modified R-Ni catalyst, it was found that catalysts modified with α-amino or α-hydroxy dicarboxylic acids have higher asymmetric hydrogenation activities than do those modified with α-amino or α-hydroxy monocarboxylic acids. That is, the asymmetric activity of the catalyst modified with aspartic acid, glutamic acid, or malic acid is higher than that of the catalyst modified with alanine, butyrine, norvaline, or lactic acid.[1-6]

The expectation that the excellent property of α-amino or α-hydroxy dicarboxylic acid as a modifying reagent will greatly depend on the ω-carboxyl group is also supported by the fact that the optimum asymmetric activity of the catalyst modified with α-amino or α-hydroxy dicarboxylic acid is produced by the modification at pH 5, which is the point where the ω-carboxyl group is neutralized. The contribution of the ω-carboxyl group to the increase in the asymmetric activity of the catalyst may be ascribable to its ionic and steric effects and to its electronegativity. Therefore, it can be expected that more acidic, more electronegative, and bulkier substituents than the carboxyl group, such as the sulfonyl group, will increase the asymmetric activity of the catalyst.

In the present work, the abilities of L-α-hydroxy-β-sulfopropionic acid, L-homocysteic acid, and L-cysteic acid as modifying reagents were tested at various modifying pH values in order to test the expectation mentioned above. The results are shown in Figs. 1 and 2, where they are compared with those obtained with aspartic acid and malic acid.

As may be seen in Figs. 1 and 2, unexpected results were obtained. The asymmetric activities of the catalyst modified with the sulfonic acids were considerably lower than those of the catalysts modified with

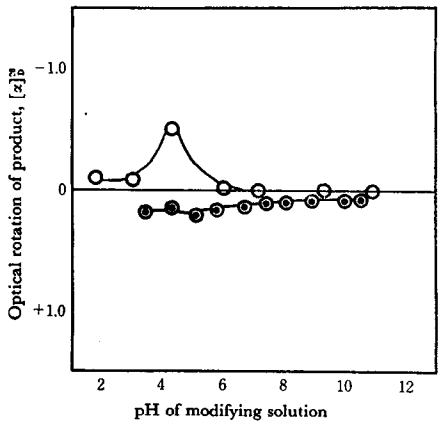

Fig. 1. Modifications with L-cysteic acid and L-homocysteic acid (Modified at 0°C).
—⊙— L-Cysteic acid $HO_3SCH_2CHCO_2H$ (NH_2)
—○— L-Homocysteic acid $HO_3SCH_2CH_2CHCO_2H$ (NH_2)

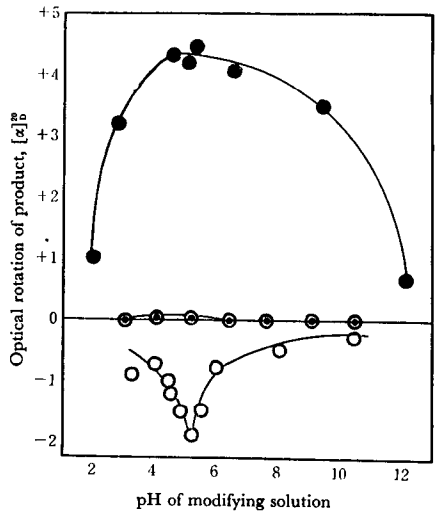

Fig. 2. Modifications with L-malic acid and L-aspartic acid and L-α-hydroxy-β-sulfopropionic acid.
-●- L-malic acid $HOOCCH_2CHCO_2H$ (OH)
-○- L-aspartic acid $HOOCCH_2CHCO_2H$ (NH_2)
-◎- L-α-hydroxy-β-sulfopropionic acid $HO_3SCH_2CHCO_2H$ (OH)

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the α-amino or α-hydroxy dicarboxylic acids. Scarcely no effect of the modifying pH is found in the case of modifications with cysteic acid and α-hydroxy-β-sulfopropionic acid, although a slight effect was found in the case of modification with homocysteic acid.

The asymmetric directions of the catalysts modified with L-homocysteic acid and L-α-hydroxy-β-sulfopropionic acid come under the general rule of the relation between the absolute configuration of the modifying reagent and the asymmetric direction of the catalyst —the catalysts modified with L-α-amino acids or D-α-hydroxy acids at 0°C produce predominantly methyl D-3-hydroxybutyrate in the hydrogenation of methyl acetoacetate. However, cysteic acid gives a catalyst which has an asymmetric direction, contrary to the general rule, as was also found in the case of the catalyst modified with serine, cysteine, and threonine.[7]

From the results described above, the regular correlations between the asymmetric activities of the catalysts modified with sulfonic acids and the ones modified with amino dicarboxylic acids were difficult to be found, from the simple view point of ionic effect, electronegativity or steric hindrance of the β- or γ-substituent.

As it was reported in the previous paper that the modifying reagent might be adsorbed on the catalyst surface with the chelate formation,[8] the contribution of the sulfonyl group to the chelate formation must be discussed. In connection with the sulfonyl group, however, it is generally known that, as the dissociation constant of the sulfonic acid is much larger than that of the carboxylic acid, the ability of the chelate formation with the metal ion is considerably weaker than that of the carboxyl group.

Accordingly, it is hard to accept the idea that the unexpected results obtained with the catalysts modified with the β- or γ-sulfonyl substituted amino and hydroxy acids are brought about by the different types of adsorption of the modifying reagent.

Therefore, it can be expected that the amino acids or hydroxy acids which have a sulfonyl group on the β- or γ-carbon are adsorbed by amino and carboxyl groups or by hydroxy and carboxyl groups.

As a conclusion of the present work, it was made clear that the electronegativity and bulkiness of the sulfonyl group on the β- or γ-carbon of the amino or hydroxy acid did not simply affect the asymmetric activity of the catalyst, and that the other new effect of the sulfonyl group overcame the effects of the electronegativity and bulkiness of the sulfonyl group.

The new effect of the substituent will be discussed in detail in This Bulletin in the near future.

The L-α-hydroxy-β-sulfopropionic acid was prepared from L-cysteic acid and was successively purified as benzidine and dicyclohexylamine salts.

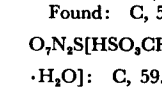
Experimental

The asymmetric activity of the catalyst was measured by a method reported in a previous paper.[6]

Preparation of the Dicyclohexylamine Salt of L-α-Hydroxy-β-sulfopropionic Acid. In 150 m*l* of 10% hydrochloric acid, 18.7 g of L-cysteinic acid monohydrate was dissolved. Into this solution, 50 m*l* of isoamyl nitrite was vigorously stirred, drop by drop, at room temperature, and then the reaction mixture was stirred continuously overnight. The isoamyl alcohol thus separated was removed, and the aqueous layer was washed thoroughly with ether. The aqueous solution was evaporated to a syrup, and the resulting syrup was taken up in a small amount of water and again evaporated. This syrup gave, quantitatively, benzidine salt in an alcohol solution; mp 260°C.

Found: C, 50.54; H, 4.71; N, 7.88%. Calcd for $C_{15}H_{18}O_6N_2S$: C, 50.85; H, 5.12; N, 7.91%.

To 17 g of syrup dissolved in 100 m*l* of acetone, was added 35 g of dicyclohexylamine, drop by drop, with ice cooling. The dicyclohexylamine salt thus precipitated was collected and washed with acetone. Two recrystallizations from ethanol-ether (1:5) gave 29.5 g (53.6%); mp 260°C.

Found: C, 58.83; H, 9.59; N, 5.22%. Calcd for $C_{27}H_{54}O_7N_2S[HSO_3CH_2CH(OH)\cdot CO_2H\cdot 2$  $\cdot H_2O]$: C, 59.00; H, 9.82; N, 5.09%. $[\alpha]_D$ −8.57 (*c* 2.3 EtOH).

Preparation of a Modifying Solution of L-α-Hydroxy-β-sulfopropionic Acid. In 30 m*l* of water, 5.36 g of the dicyclohexylamine salt was dissolved. The dicyclohexylamine was removed using a column of Amberite IR 120(400—600 mesh, 1×18 cm), and with water. The total volume of the eluted solution was adjusted to 100 m*l*. The specific rotation $[\alpha]_D$ −13.3 (*c* 1.65, H_2O) for L-α-hydroxy-β-sulfopropionic acid was calculated from the α_D of the solution obtained. An attempt to isolate the free acid failed.

The authors wish to express their thanks to Professor Shōichirō Yamada and Professor Yōichi Shimura, Osaka University, for their helpful discussions, and to the Ajinomoto Co., Ltd., Tokyo, for the gift of L-homocysteic acid.

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7) Proline, hydroxyproline, and alanine all produced catalysts which have an asymmetric direction, thus contradicting the general rule. However, proline and hydroxyproline are special amino acids which have a pyrrole ring, and the modification with alanine is very sensitive to the modifying condition, so such results are reasonable even according to the general rule. The details of the asymmetric activity of the catalyst modified with alanine will be discussed in This Bulletin in the near future.

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