PHYSICO-CHEMICAL STUDIES ON THE SOLUBLE COLLAGEN OF RAT-TAIL TENDON

by

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It has long been known[1] that tendon could be dissolved in dilute acid, and could be reprecipitated as a fibrous substance which had the regular structure of the original substance. Later it was found that the reconstituted fibers possessed a regular structure even when examined by X-ray diffraction[2,3] and the electronmicroscope[4]. The re-reprecipitation can be carried out by a change of pH of the solution or by the process of salting out, and the conditions were elaborated by WYCKOFF and the writer[5]. Recently fibrous substances of various spacings and various shapes which are not present in nature have been precipitated from collagen solution[6,7].

Since the dissolution from tendon into a dilute acid solution takes place very rapidly in a few minutes, and it is impossible to find any fibrous pieces in the solution with an electronmicroscope, and since centrifugation in a field 40,000 times the gravity does not make any change in the ability of the solution to reprecipitate collagen fibers, it is assumed to be a molecular solution.

In order to discover more about the shape and size of molecules in a solution obtained by extracting tendon with dilute acid solution, the solution was purified to some extent and then its physicochemical properties such as sedimentation constant, viscosity and diffusion constant, were measured.

EXPERIMENTAL

Material. Collagen solution was prepared by extracting freshly excised tail tendon of albino rats of about 150 grams of body weight. After the tail was cleaned and its skin was peeled, each individual tendon was pulled with a pair of sharp pointed tweezers, cut at the place of its attachment to the bone, and immediately immersed in a solution of one part of glacial acetic acid in 5,000 parts of distilled water. In this way very clean tendon was obtained and the gelatinous matter which occasionally accompanied tendon was avoided. It was left to stand in a refrigerator over night, and then filtered with paper using an aspirator. One volume of 0.1 *M* sodium citrate buffer of pH 4.7 was added to three volumes of the extraction solution, making the final pH between 4.6 and 4.8. The solution was left to stand in a thermostat kept at a temperature of 20° C for a few hours, and then the precipitate was collected by low speed centrifugation. The supernate was again left to stand for a few hours in the thermostat of 20° C, and the precipitate thus formed was joined to the previous precipitate. The precipitate was again dissolved in a solution of one part of glacial acetic acid in 5,000 parts of distilled water, and then precipitation was performed with citrate buffer in the same way as before. This procedure of precipitation and solution was repeated several times.

The final solution thus obtained was dialyzed against distilled water at a temperature of 1° to 2° C for two to three days. Then the solution was lyophilized. Yield was about 20 mg from one rat. Such dried collagen could be stored in a refrigerator without losing its ability to reprecipitate.

In order to prepare a solution for the following measurements, collagen dried *in vacuo* over P_2O_5 was weighed and dissolved in a measured volume of distilled water to which one part in 5,000 of glacial acetic acid had been added, and the solution thus obtained was dialyzed against sodium acetate buffer of $\Gamma/2 = 0.1$ and $pH = 4.0$ for a few days at 1° to 2° C. The concentration of the final solution was checked by nitrogen determination, and it was verified that the dialysis did not cause any appreciable change in concentration. Immediately before each measurement, the solution was centrifuged in an air-driven centrifuge with a field of 30,000 *g*, in order to remove minute amounts of foreign particles.

Sedimentation measurement. Sedimentation experiments were performed on various dilutions of solution using a Spinco Model E ultracentrifuge. A speed of 59,780 revolutions per minute was used, and the field was 260,000 times that of gravity. Photographs were taken at 16 minutes intervals.

Viscosity measurement. Viscosity measurements were performed with capillary viscometers dipped in a thermostat kept at a temperature of 20° C. The capillary viscometers used were designed by Dr. YOSHIMI KAWADE[8] of this Institute and have a low velocity gradient of flow of about 100 sec^{-1} which makes the effect of molecular orientation negligible. The water constant of the viscometers was around 100 seconds.

Diffusion measurement. Measurements were performed refractometrically by the SVENSSON schlieren method[10] using a NEURATH type[9] cell.

Amino acid component determination. Collagen was hydrolyzed with HCl at 107° C for 40 hours, the hydrolyzate was examined with 2-dimensional paper chromatography. Phenol-ammonia-hydroxy quinoline was used for the first development and butanol-acetic acid-water mixture was used for the second development. In order to develop color ninhydrin was used.

Measurement of partial specific volume. Density of distilled water, buffer and solutions of collagen was measured with glass pycnometers of 3 to 5 ml capacity. Weight was measured with a micro-balance.

RESULTS

Purification. When the nitrogen content of the first precipitate from the extraction solution of tendon was measured by micro-Kjeldahl method, it was about 18% in every case, but it became 15% by redissolving the precipitate and reprecipitating with citrate buffer. The nitrogen content stayed at 15% thereafter throughout successive redissolutions and reprecipitations. The extraction solution of tendon reduced Fehling's solution when it was hydrolyzed with sulfuric acid. The precipitate formed by the addition of citrate buffer did not reduce Fehling's solution when it was hydrolyzed with sulfuric acid, while the mother liquor reduced it upon hydrolyzing with sulfuric acid.

When the purified collagen solution was observed while centrifuged in a field of

260,000 *g*, a single sharp boundary was observed regardless of the concentration of the solution. (from 0.016% to 0.3%) (*cf*. Fig. 1.).

Amino acid composition. Amino acids found are arranged as follows, in the order of ninhydrin color reaction.

glycine > leucine + phenylalanine, aspartic acid, alanine, glutamic acid, valine > arginine, serine > histidine (trace), tyrosine(trace); proline, hydroxyproline.

Proline and hydroxyproline were found in considerable amounts, but their amounts could not be compared with others due to different color reaction with ninhydrin.

Sedimentation constant. Sedimentation experiments were performed on 4 independently prepared batches of pure preparations. For each preparation 3 to 4 different dilutions were used. Results obtained are shown in Fig. 2. As is clearly seen in Fig. 2, the sedimentation constant was dependent on concentration. When the reciprocal number was plotted against the concentration, the points did not lie on a straight line. Sedimentation constant values multiplied by respective values of the solution viscosity did not lie on a straight line when plotted against concentration. Thus the sedimentation constant at zero concentration was estimated by a manual extrapolation on Fig. 2. The value obtained was $S_{20,w} = 3.5 \cdot 10^{-13}$ cm/sec dyne.

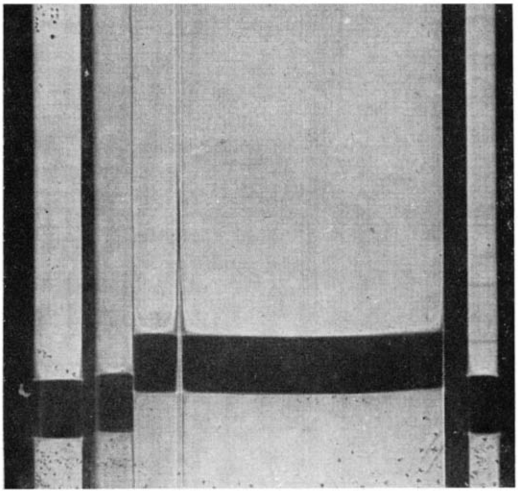

Fig. 1. Sedimentation pattern of soluble collagen. Spinco Model E ultracentrifuge was used. Preparation No. 3. Concentration 0.17%. Sodium acetate buffer, pH 4.0, $\Gamma/2 = 0.1$.

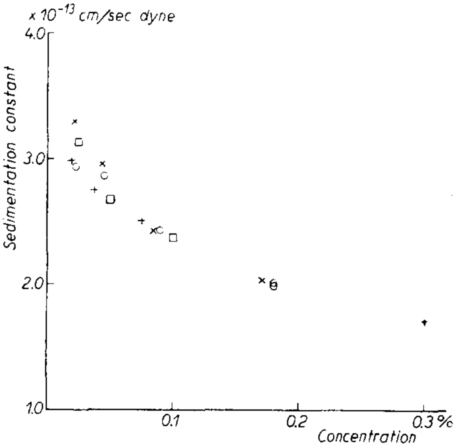

Fig. 2

○ Preparation No. 1
× Preparation No. 3
□ Preparation No. 4
+ Preparation No. 5

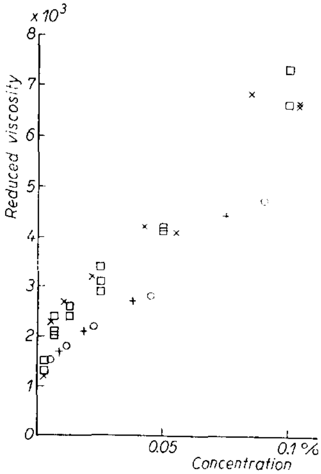

Fig. 3

○ Preparation No. 1
× Preparation No. 3
□ Preparation No. 4
+ Preparation No. 5

References p. 98.

Viscosity. Values of reduced viscosity (specific viscosity divided by volume fraction concentration) obtained are shown in Fig. 3. Two sets of values are obviously smaller than the other two sets of values, which may indicate that there was some degradation of molecules during the course of purification. By extrapolating values in Fig. 3 to zero concentration, $1.5 \cdot 10^3$ was obtained as the value of intrinsic viscosity. Using the formula proposed by SIMHA[11] for prolate ellipsoids of revolution, the axial ratio of the collagen molecule was calculated to be 170. From this value of a/b the frictional ratio for collagen molecules was calculated to be 5.25 using the formula proposed by PERRIN[12].

Diffusion constant. Due to the grease-emulsifying property of collagen solution and the difficulty in obtaining enough concentration, successful experiments were carried out only for preparations No. 3 and No. 5. The pattern obtained showed a considerable degree of skewness. In the experiment for preparation No. 3 the concentration of solution was 0.17%, and was too small to provide a reliable value. The diffusion constant obtained from the experiment for preparation No. 3 and reduced to zero concentaration was $0.33 \cdot 10^{-7}$ cm^2/sec. In the case of preparation No. 5, the concentration was 0.3%. The diffusion constant was calculated by moment method[13]. The diffusion constant at infinite dilution was calculated according to the method of GRALÉN[14]. The obtained value was $D_{20,w} = 0.5 \cdot 10^{-7}$ cm^2/sec.

TABLE I

Preparation	*Partial specific volume*
No. 1	0.77_5
No. 4	0.78_0
No. 5	0.78_3
Average	0.78

Partial specific volume. Values of partial specific volume measurements are shown in Table I.

Molecular weight and shape estimation. The molecular weight and frictional ratio f/f_0 were calculated from measured values of sedimentation and diffusion constants according to the method of SVEDBERG[15]. Axial ratio a/b ($2a$ and $2b$ are long and short axes of molecules) was calculated by the method of PERRIN[12] assuming that molecules were prolate ellipsoids of revolution. Obtained values were

$$M = 7.1 \cdot 10^5, \quad f/f_0 = 6.9, \quad 2a = 5.2 \cdot 10^3 \text{ A}, \quad 2b = 18 \text{ A}.$$

Since axial ratio and frictional ratio of particles can be calculated from the viscosity of solution as was described in the previous section, it is possible to obtain values of molecular weight and size using values of viscosity. When the molecular weight calculated from values of sedimentation and diffusion constants and axial ratio calculated from the viscosity value were used, dimensions of the molecule were calculated to be $2a = 3.7 \cdot 10^3$ A and $2b = 22$ A. When calculated from values of sedimentation constant and f/f_0 from viscosity, a molecular weight of $5.1 \cdot 10^5$ was obtained. From values of diffusion constant and f/f_0 from viscosity $1.7 \cdot 10^6$ was obtained for the molecular weight.

DISCUSSION

Since the mechanism and components participating in the reprecipitation of striated fibers from the extraction solution of tendon is not known, it is uncertain whether the substance used in experiments described can be called collagen or not. There was some evidence revealed by nitrogen determination and reducing reaction that certain substances were eliminated in the process adopted as purification process. Judging from the pattern observed in sedimentation experiment, collagen solution consisted of a homogeneous component. However, it is not possible to exclude the possibility of the presence of other minor components. In any case, the protein component of the extraction solution of tendon which is called collagen in the present report is the main component of striated fibers of collagen.

When Fig. 2 was carefully examined, experimental points for dilute solutions fell roughly into two groups, Except in two single cases points for preparations No. 3 and No. 4 belonged to the group of higher values, and points for preparations No. 1 and No. 5 belonged to the group of lower values. In results of viscosity measurements there was also a tendency which conformed with the above observation in sedimentation experiments. As is seen in Fig. 3, values for preparations No. 3 and No. 4 formed a separate group of higher values. Hence it seems that degradation due to some unknown cause took place in preparations No. 1 and No. 5. However, there were two points observed in sedimentation experiments which did not fit in the separation in two groups, and three points of viscosity measurements for the most dilute solutions of preparations No. 3 and No. 4 did not fall on the straight line through other points of preparations No. 3 and No. 4. Although it was uncertain whether it was due to experimental errors or to molecular degradation, differences in measured values in sedimentation constant and viscosity did not make a significant difference in calculated values of molecular weight and molecular shape.

The difference in two values obtained for diffusion constant was rather significant in molecular weight calculation. If it is assumed that some degradation took place in preparation No. 5, the difference in diffusion constant was qualitatively in accordance with the difference in sedimentation and viscosity values. However, the difference in values of diffusion constant was too large. The difference in diffusion constant large enough to explain differences in sedimentation and viscosity values for degraded and non-degraded molecules was calculated to be only 10%. Due to the skewness of curves obtained in diffusion experiments, more error was probable for the experiment with preparation No. 3 in which the concentration was not high enough to make a sufficiently large boundary figure to be accurately measured. Therefore the value of diffusion constant for preparation No. 5 was used in the molecular weight calculation.

In calculations in the previous section, hydration of collagen molecules in the solution was not taken into consideration. According to the report by BULL[16] and WEIR[17] hide powder collagen adsorbed about 50% water at about 95% relative humidity. It appeared that more than 50% in weight of water was adsorbed at 100% relative humidity when the curve was extrapolated. The material obtained in this experiment was also very hygroscopic. Therefore a calculation of molecular size by assuming 50% hydration was attempted. The molecular weight of non-hydrated molecules was taken as $7.1 \cdot 10^5$. By 50% hydration the weight of each particle should be $1.07 \cdot 10^6$, and the volume of each molecule should become 1.64 times larger. Using Stokes' law the ratio of friction for a sphere the volume of which is equal to a hydrated molecule, to friction for a sphere the volume of which is equal to a non-hydrated molecule, was calculated to be 1.18. The frictional ratio f/f_0 obtained from sedimentation and diffusion constants was 6.9. Therefore the ratio of friction for the hydrated molecule, to friction for a sphere the volume of which is equal to a hydrated molecule, became 5.8. Using PERRIN's[12] equation, the axial ratio of hydrated molecule was calculated to be 205. Then dimensions of 50% hydrated molecule were calculated as $2a = 5.0 \cdot 10^3$ A and $2b = 25$ A. When we compare this value with that obtained from values of sedimentation and diffusion constants without assuming hydration, the effect of hydration is mainly to increase the width of long molecules.

Throughout treatments described above, molecules were always assumed to be rigid ellipsoids of revolution. A very long and thin shape was obtained as the result. It is difficult to imagine that such long molecules are rigid in solution. In addition, there are some uncertainties concerning the homogeneity of collagen used and the accuracy of diffusion constant measurement. However, when factors mentioned above were carefully examined, it was found that values calculated in the above could not be far from the true values.

It is well known that collagen fibers have regular striations at 650 A spacings when examined under an electronmicroscope[18,19,20]. Striations at a spacing of *ca* 215 A have also been occasionally observed[5,21]. Solution of collagen extracted from rat tail tendon and purified in the way described in the above yielded long-spacing fibers[22] such as observed by OREKHOVICH *et al.*[23] upon addition of bovine serum glycoprotein to suitable concentrations (without dialysis). HIGHBERGER, GROSS AND SCHMITT[24] studied the distribution of axial periods of such kind of fibers and found that it ranged from 2000 to 3000 A. Recently SCHMITT, GROSE AND HIGHBERGER[7] found a new segment type long-spacing fibers precipitated from connective tissue extract solution. The average period of such kind of segment long-spacing fibers was between 1700 A to 2500 A according to the difference of the source of connective tissue.

By X-ray diffraction measurements on tendon, it was reported that equatorial spacings of collagen fibrils vary from 10 A to 15 A due to various degree of hydration[25,26].

Values of spacings cited above do not seem to have any relation with the values of size of collagen molecules obtained here. Further study must be done before any connection between the molecules and striations is clarified.

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SUMMARY

From an extraction solution of rat-tail tendon with dilute acetic acid, a major protein component which was assumed to be responsible for reprecipitating striated fibers was purified. By measuring sedimentation constant, viscosity and diffusion constant, its molecular weight and shape were calculated. The molecular weight was found to be about 700,000. Assuming the molecules to be prolate ellipsoids of revolution, the long axis was calculated to be 5,000 A and the short axis 18 A.

RÉSUMÉ

A partir des produits d'extraction par l'acide acétique dilué du tendon de la queue de rat, les auteurs ont purifié un constituant protéique important, probablement capable de donner des fibres striées par reprécipitation. La mesure de la constante de sédimentation et des constantes de viscosité et de diffusion a servi à déterminer le poids moléculaire et la forme de la molécule. Ce poids moléculaire est voisin de 700,000. Les molécules seraient des ellipsoïdes de révolution allongés, dont le grand axe mesurerait 5,000 A et le petit axe 18 A.

References p. 98.

ZUSAMMENFASSUNG

Aus der Extraktionslösung der Rattenschwanzsehne mit verdünnter Essigsäure, wurde eine grössere Protein Komponente rein dargestellt, die für die Repräcipitation quergestreifter Fasern verantwortlich sein soll. Durch Messung der Sedimentations- und Diffusionskonstanten wie der Viskosität wurde das Molekulargewicht und die Gestalt berechnet. Das Molekulargewicht wurde zu 700,000 gefunden. Unter der Annahme, dass die Moleküle gestreckte Rotationsellipsoide sind, wurde eine lange Achse von 5000 A und eine kurze Achse von 18 A berechnet.

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