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The Native and Denatured States of Soluble Collagen

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A number of physical methods have been used to show that soluble collagen prepared by citrate extraction of carp swim bladder tunics (ichthyocol) consists of rigid, rod-shaped molecules having a very narrow size distribution. The diameter is 13.6 A., and the weight average molecular length and weight are 3000 Å. and 345,000, respectively. This very asymmetric molecule undergoes denaturation in dilute salt solution at about 30° with an activation energy of 81 kcal. to form a product known as parent gelatin. However, the tendency toward reaggregation below this temperature and the significant rate of hydrolysis above this temperature (energy of activation 24 kcal.) creates unusual difficulty in characterizing the denatured state. The demonstration that 2 *M* KCNS lowers the denaturation temperature below 4° makes possible the avoidance of reaggregation by the use of this solvent. The number and weight average molecular weights were then shown to be 125,000 ± 10,000 and 138,000 ± 8000, respectively. The ratio of the molecular weight of collagen to the denatured form (parent gelatin) was found to be 2.5 ± 0.15 in separate experiments involving heating directly in the light scattering cell. The interpretation of these data shows that three molecules are formed from each collagen molecule upon denaturation, but the ratio of 2.5 requires that the molecules be of substantially different molecular weights. The dimensions of the collagen molecule are so much larger than the main long spaced periodicity (700 Å.) in collagen fibrils that several, probably four, of these must be contained within each molecule. Moreover, the size of the average parent gelatin molecule is such that it can traverse the whole length of the collagen molecule. A comparison with the three-stranded collagen structures recently deduced from X-ray studies shows that the observed mass-to-length ratio of 110 is sufficiently close to the required value and that the observed diameter is in excellent agreement. The conclusion therefore follows that the soluble collagen molecule is composed of three polypeptide strands running lengthwise and that it does serve without serious modification as the structural element of collagen fibrils. Moreover, this molecule has the additional characteristics of a hypothetical particle, tropocollagen, which Schmitt, Gross and Highberger concluded must exist as the common building unit of the three forms of collagen observed electron microscopically. Finally, suggestions are made of ways in which the three chains may be mutually arranged in the molecule so as to provide for a dangling chain at one end and a double chain at the other. These rather unique arrangements permit a closer fit of our data and a basis of constructing polarized protofibrils without the need of a continuous primary bonded framework.

Although the protein collagen exists in nature in the form of partially crystalline, microscopically visible fibers, it has been known for more than half a century that an appreciable fraction of these fibers can be solubilized under relatively mild conditions.[2] By 1927 Nageotte had demonstrated[3] that fibers having the microscopic appearance of native collagen occurred in the precipitates from such solutions. Despite the implication from this work that biological structures were being reconstituted from solution, 20 years passed before attempts were made to examine the physical chemical properties of collagen in solution. Meanwhile it was observed that the X-ray diffraction features of the reconstituted fibers were similar to those of the native fibers.[4] Following the discovery of the long range periodicity of approximately 640 Å. in collagen fibrils (the component strands of the fibers) by low angle X-ray diffraction[5] and by direct visualization in the electron microscope,[6] this "fingerprint" was found in the reconstituted fibrils.[7]

The examination of collagen solutions by biophysical methods began with the report of Bresler, *et al.*[8] who found molecular weights of about 70,000 for a soluble skin collagen prepared by Orekhovich, *et al.*[9] More recently Mathews, *et al.*,[10] reported for similar material a number average molecular weight of 74,000 from osmotic pressure and a weight average value from light scattering of the order of 10,000,000. These authors indicated that the number average value was much too low to be consistent with the observed values of sedimentation and viscosity and suggested that large aggregates were present. Light scattering molecular weights of several million were also found by Veis, *et al.*,[11] for soluble bovine skin collagen extracted at 60°. Another light scattering investigation of soluble collagen by M'Ewen and Pratt[12] yielded molecular weights of about 7 million for extracted skin collagen and from 10 to 25 million for tendon. Their interpretations suggested that the soluble units were thread-like in shape and of extreme length, about 100,000 Å. The diversity of these results certainly indicated the presence of both light and heavy macromolecular particles.

In 1953 we became acquainted with the work that Gallop was carrying out in Professor R. S. Bear's Laboratory. Gallop adapted the citrate extraction procedure of Orekhovich to the tunics of the swim bladder of the carp to prepare very pure collagen solutions. Sedimentation, viscosity and light scattering studies led him also to conclude that the soluble units were long and thread-like but with molecular weights of only one to two million.[13] In addition he showed that this material underwent a dramatic, irreversible change when heated at *p*H 3.7 to only 40°. The product, called parent gelatin, was shown to have a molecular weight of 70,000.[13] This discovery appeared to explain the discrepancies in the previous studies because either

(1) Milton Fellow, 1954–1955.

(2) P. A. Zacchariades, *Compt. rend. soc. Biol., Paris*, **52**, 182, 1127 (1900).

(3) J. Nageotte, *Compt. rend. Acad. Sci., Paris*, **184**, 115 (1927).

(4) R. W. G. Wyckoff and R. B. Corey, *Proc. Soc. Exptl. Biol. Med.*, **34**, 285 (1936).

(5) R. S. Bear, THIS JOURNAL, **64**, 727 (1942).

(6) C. E. Hall, M. A. Jakus and F. O. Schmitt, *ibid.*, **64**, 1234 (1942)

(7) F. O. Schmitt, C. E. Hall and M. A. Jakus, *J. Cellular Comp. Physiol.*, **20**, 11 (1942).

(8) S. E. Bresler, P. A. Finogenov and S. Y. Frenkel, *Reports Acad. U.S.S.R. Moscow*, **72**, 555 (1950).

(9) V. N. Orekhovich, A. A. Tustanovskii, K. D. Orekhovich and N. E. Plotnikova, *Biokhimiya*, **13**, 55 (1948).

(10) M. B. Mathews, E. Kulonon and A. Dorfman, *Arch. Biochem. Biophys.*, **52**, 247 (1954).

(11) A. Veis, D. N. Eggenberger and J. Cohen, THIS JOURNAL, **77**, 2368 (1955).

(12) M. B. M'Ewen and M. I. Pratt, "Nature and Structure of Collagen," edited by J. T. Randall, Academic Press, New York, N. Y., 1953, pp. 158–168.

(13) P. M. Gallop, *Arch. Biochem. Biophys.*, **54**, 486, 501 (1955).

the high molecular weight form or the parent gelatin or both could exist in a given case depending on the thermal history of the solution. One particular contradiction remained, however. The intrinsic viscosities of the various samples studied by light scattering[12,13] were of nearly constant value (13 to 18 dl./g.) in contrast to the 20-fold variation in molecular weights.

During this same period Schmitt, Gross and Highberger[14,15] succeeded in preparing from solution and identifying by means of electron microscopy two entirely different forms of collagen fibrils. Since these were interconvertible with the native form, they concluded that collagen solutions must contain a fundamental unit capable of generating all three fibrillar forms. Since the lengths of one of these forms did not exceed 3,000 Å., it seemed possible that this represented the upper limit for the length of the fundamental unit. This stands in conflict with the contour lengths of 4 to 50 times 3,000 Å. assigned on the basis of light scattering studies.[12,13]

In the hope that the foregoing contradictions could be resolved, a new investigation was undertaken. In addition to possibly improving the characterization of soluble collagen, we were interested in studying its thermal denaturation in relation to the denaturation of globular proteins and in finding out the structural relation of the polypeptide strands of parent gelatin to the soluble collagen particle.

After a summary of the preparative and physical chemical methods employed, this report is divided into three parts: the characterization of soluble collagen,[16] the denaturation of soluble collagen and the characterization of the denatured state and its relation to the soluble collagen.

Experimental Methods

Preparation and Handling of Soluble Collagen (Ichthyocol).—The *tunica externa* of fresh carp swim bladders were treated by the method described by Gallop.[13] This involved homogenization and extractions with sodium acetate solutions followed by extraction of the remaining tissue paste with cold *p*H 4.3 citrate buffer. The extract was ultracentrifuged and then dialyzed against 0.02 *M* dibasic sodium phosphate. Rigid, needle-shaped fibrils formed in the dialysis bag and were collected, washed and stored wet at −10° or immediately dissolved. Successive preparations were labeled A, B, etc.; successive extractions in a given preparation were denoted as A-1, A-2, etc. If the product of a given extraction was dissolved and reprecipitated by repetition of the dialysis against phosphate, the products were denoted as A-1-1, A-1-2, etc.

All solutions were made up by allowing the precipitate to dissolve in citrate buffer (0.1 *M* citric acid, 0.05 *M* sodium citrate, *p*H 3.7) at 4° for about 20 hr. and then dialyzing the resultant solution against the same buffer for 12 hr. Thereafter the temperature of the solution was never permitted to rise above 20°; it was kept at 4° as much of the time as possible.

Concentration Determinations.—Concentrations were determined by means of a biuret procedure[17] which was calibrated against micro-Kjeldahl determinations. Gallop's[13] determination of 17.5% nitrogen was used to convert nitrogen analyses to dry weight of collagen. Reproducibility of the biuret method was ±2%.

Intrinsic Viscosity.—Collagen solutions were found to have the same relative viscosity in special multigradient viscometers[18] with gradients in the range of 70 to 200 sec.$^{-1}$ as in high gradient Ostwald–Fenske viscometers having water flow times of 200 to 250 sec. provided the concentration was less than 0.04 g./100 cc. Measurements were therefore made in Ostwald–Fenske viscometers. For collagen solutions temperatures of 15° were used although some measurements at 20° gave the same result for the intrinsic viscosity.

Flow Birefringence.—The extinction angle, χ, and the birefringence, Δn, were measured on a Rao Instrument[19] over a gradient range from 800 to 6000 sec.$^{-1}$ in a room thermostated at 20 ± 1°. Concentration dependence of χ and $\Delta n/c$ vanished below 0.1 g./100 cc. and therefore only results obtained at concentrations below this limit are reported.

Sedimentation Constant.—Sedimentation was observed in a Spinco Model E Ultracentrifuge. Refrigeration was used for all measurements on collagen solutions in order to maintain the temperature below 20°. The value of the sedimentation constant (uncorrected) was calculated from a plot of the log of the displacement at time t against $\omega^2 t$, where ω is the angular velocity. The temperature was taken as the average of the rotor temperature at the beginning and end of the run. The sedimentation constant, s, was then corrected to that for water at 20° by multiplying by the two viscosity ratios, $\eta_t/\eta_{20°}$ and $\eta_{solvent}/\eta_{H_2O}$ and by $(1-\bar{v}\rho^{20}_{H_2O})/((1-\bar{v}\rho^{t}_{solvent})$.

Partial Specific Volume.—The partial specific volume of parent gelatin was measured in 2 *M* KCNS at 25° using a 10-ml. pycnometer. The value was found to be 0.695 ± 0.005. This is in agreement with Gallop's value of 0.705 ± 0.005 in citrate buffer. The high viscosity of collagen solutions prevented the removal of entrapped air to such a degree that satisfactory measurements could not be made.

Osmotic Pressure.—Osmotic measurements of collagen solutions were made with a simple Adair osmometer using cellulose nitrate membranes prepared by Adair's method.[20] Because of the high viscosity and difficulty in measuring the capillary rise of these solutions 2 mm. diameter capillaries were employed. The rise was independent of concentrations over the range investigated (0.25 to 0.68 g./100 cc.) and had a value of 1.15 ± 0.05 cm. The uncertainty in this quantity made the largest contribution to our estimated error. The measurements themselves were reproducible within ±5%. Equilibrium was reached within 24 hr.

Osmometers of the Bull design[21] were employed for the parent gelatin solutions. The same type of membranes were used. The appropriate density corrections were made after density determinations of both the solution and the toluene had been made in 25-ml. pycnometers.

Light Scattering.—Light scattering measurements were made in a slightly modified Brice–Speiser photometer in a manner that has been described previously.[15,22] Both erlenmeyer flask-shaped cells and cylindrical cells were used. Solutions and solvent were clarified by ultracentrifugation. In the course of the examination of collagen solutions it became apparent that the exceedingly high relative viscosity of solutions that were more concentrated than 0.2 g./100 cc. prevented their clarification by ultracentrifugation. As a consequence clarification was carried out below this limit and measurements were made in the range 0.02 to 0.07 g./100 cc.

Refractive Index Increment.—The specific refractive index increment, dn/dc, was determined with a Brice–Speiser differential refractometer. Solutions were first exhaustively dialyzed against solvent, their concentration measured and one or more dilutions made up gravimetrically. The value of dn/dc of collagen and gelatin in citrate buffer were found to be the same, 0.187 ± 0.004. In 2 *M* KCNS the value for gelatin was 0.173 ± 0.003.

(14) F. O. Schmitt, J. Gross and J. H. Highberger, *Proc. Natl. Acad. Sci.*, **39**, 459 (1953).

(15) J. Gross, J. H. Highberger and F. O. Schmitt, *ibid.*, **40**, 679 (1954).

(16) A preliminary report of this part has appeared; H. Boedtker and P. Doty, This JOURNAL, **77**, 248 (1955).

(17) G. A. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).

(18) A. M. Holtzer, H. Benoit and P. Doty, *J. Phys. Chem.*, **58**, 624 (1954).

(19) J. T. Edsall, A. Rich and M. Goldstein, *Rev. Sci. Instr.*, **23**, 695 (1952).

(20) See, for example, H. Gutfreund, *Trans. Faraday Soc.*, **50**, 684, 688 (1954).

(21) H. Bull, *J. Biol. Chem.*, **137**, 143 (1941).

(22) H. Boedtker and P. Doty, *J. Phys. Chem.*, **58**, 968 (1954).

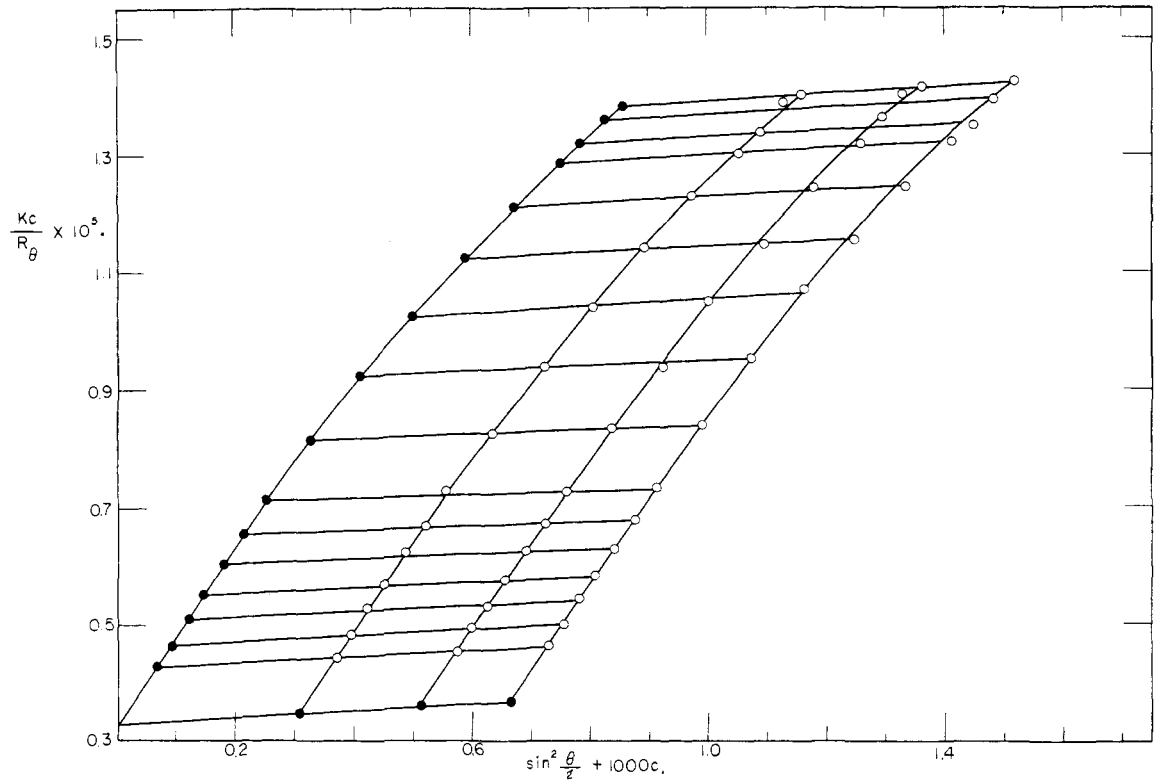

Fig. 1.—Light scattering of collagen solutions: Sample F-1, citrate buffer, *p*H 3.7, 15°; M_w = 310,000; ρ = 870 Å.; L = 3000 Å.; M/L = 103: O, experimental data; ●, extrapolated points.

The Collagen Molecule

When, as in this case, the major investigative methods of macromolecular solutions are focused on one problem, the results reflect interrelated information of varying accuracy on molecular weight, molecular weight distribution, molecular shape and the distribution of molecular shapes. We consider first the information provided by light scattering and osmometry and then that derived from hydrodynamic measurements: viscosity, sedimentation and birefringence of flow.

Light Scattering and Osmometry.—Since the greatest diversity in earlier observations had been in light scattering, we turned first to this type of observation and found likewise a tremendous variation of molecular weights (about 10-fold) while noting that the intrinsic viscosity was nearly invariant. This contradiction became resolved when it was found that optical clarification was not being produced by ultracentrifugation as long as the relative viscosity exceeded 3 (concentration of 0.2 g./100 cc.). This point is discussed in detail in Appendix I.

With this restriction on the concentration at which centrifugation took place, fairly reproducible results were obtained and from Zimm-type plots (Fig. 1) the molecular weights shown in Table I were obtained. The average value of these weight average molecular weights is 345,000 ± 30,000.

The interpretation of the downward curvature of the reciprocal scattering envelope (Figure 1) usually presents a dilemma because it can be accounted for either in terms of the degree of coiling of relatively extended, coiled configurations[12,13] or in the breadth of the distribution of lengths of rod-like particles. Upon examination of the detailed shape of the reciprocal scattering envelopes corresponding to the entries in Table I, however, it was found that a fit within a maximum deviation of less than 5% could be obtained with the scattering formulas for rods of uniform length. An example of this is shown in Figure 2 for sample I-1 where the theoretical plot for a length of 3,000 Å. is seen to fit all but the highest angles quite well. Ordinarily the dimension of the particle is obtained from the limiting slope and intercept of the Zimm-plot. When that procedure is used here, values higher by about 7% are obtained, but the fit of the theoretical curve for this higher value of the length is distinctly poorer. The reason for this lies in the fact that for rods of this length the limiting slope for the reciprocal particle scattering factor, $P(\theta)^{-1}$, lies at lower angles than those at which measurements are made. Therefore we have assigned the value of the lengths on the basis of the best fit of the low angle data. Depolarization of the scattered light was found not to affect the assignment of weight and dimension significantly; it is summarized in Appendix II.

These light scattering results indicate that the collagen molecule is quite different than previously supposed. Moreover, it is seen from Table I that within probable error the results are the same for successive extractions of the same tissue and successive precipitations of the same sample.

The good fit of the angular data with rods of uniform length would settle the problem of shape providing only that polydispersity is minimal.

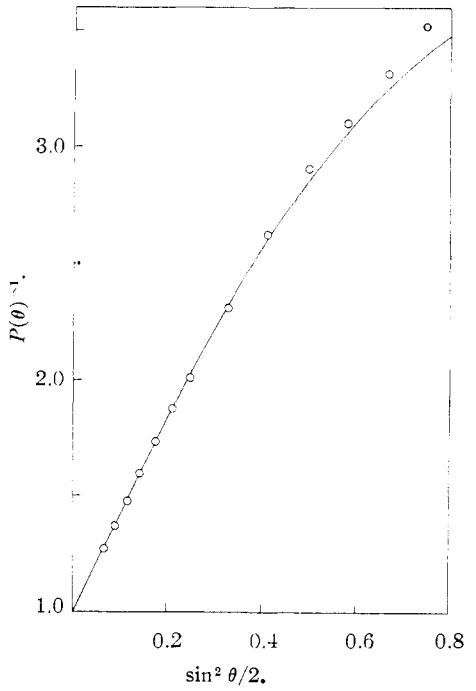

Fig. 2.—Reciprocal particle scattering factor of collagen solution: sample I-1, citrate buffer, *p*H 3.7, 20°; O, experimental points; —, theoretical scattering curve for rod 3000 Å. long.

That is, the possibility remained that the observed downward curvature reflected the scattering of very polydispersed thread-like molecules, rather than that of nearly monodispersed rods. In that case, one would expect the weight average molecular weight to be at least three times higher than the number average molecular weight. The correct interpretation of the downward curvature and the identification of the shape of collagen molecules depended therefore on obtaining an estimate of the breadth of the molecular weight distribution. For this reason osmotic pressure measurements were carried out. The results for sample F-1 are shown in Figure 3. The small pressures involved and the

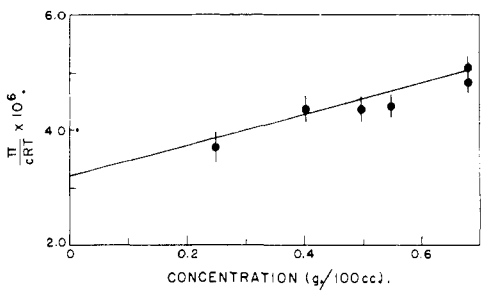

Fig. 3.—Osmotic pressure of collagen solutions: sample F-1, citrate buffer, *p*H 3.7, 2°, M = 310,000 ± 50,000

very high viscosity of the solution caused considerable inaccuracy. However, from these data we can conclude that the number average molecular weight is 310,000 ± 50,000 and that the second virial coefficient is 2.3 ± 1.0 × 10^{-4} cc. mole/g.^{2} This value lies within range of the weight average molecular weight and indicates thereby that the molecular weight distribution is extremely sharp.[23]

TABLE I

LIGHT SCATTERING AND VISCOSITY RESULTS ON COLLAGEN SAMPLES

Sample	Wt. av. mol. wt.	Length, Å.	Intrinsic viscosity	M/L
F-1	310,000	3000	13.4	103
F-2-1[a]	307,000	2950	11.1	104
F-2-2	380,000	3500	..	109
F-3-1	370,000	3350	10.0	110
H-1[a]	340,000	2850	..	119
I-1[a]	340,000	3000	..	113

[a] The light scattering was measured at only one concentration (approximately 0.05 g./100 cc.) and corrected to zero concentration using B = 3 × 10^{-4} cc. mole/g.^{2}.

Intrinsic Viscosity.—Several measurements of intrinsic viscosity are listed in Table I. These and other determinations give an average result of 11.5 ± 1.5 dl./g. Accepting the rod-like shape of the particle deduced from light scattering as well as the molecular weight, the value of the intrinsic viscosity can be used to accurately estimate the molecular diameter and length. The use of Simha's equation for the intrinsic viscosity of ellipsoids[24] and the value of the partial specific density (1/0.700 = 1.43) leads to an axial ratio of 178. An ellipsoid of revolution having this axial ratio and the cited mass (345,000) and density has a minor axis of 16.7 Å. and a major axis of 2970 Å. Since it is most likely that the geometrical form of the particle is closer to a right cylinder than an ellipsoid of revolution, it is desirable to assign the dimensions of such a cylinder. If we make the assumption[25] that the closest cylindrical equivalent has the same length and density as the ellipsoid, the diameter of the cylinder is given by $(2/3)^{1/2}$ × 16.7 = 13.6 Å. If all the variation in intrinsic viscosity were considered to lie in experimental errors and not in variable lengths of the preparation, this value of the diameter would have an uncertainty of ± 0.4 Å. On the other hand, if all the probable errors were associated with variation in average particle length, the value would be represented by 2970 ± 200 Å. If the probable error in the molecular weight were also taken into account, these uncertainties in dimensions would be nearly doubled.

It is interesting to note that the results obtained in the foregoing interpretation of viscosity are unaffected by any hydration that may exist. This insensitivity occurs at high axial ratios because the hydration shell makes a significant contribution only to the minor axis. The effect of this in turn, through the square of the axial of ratio in Simha's equation, is exactly offset by the corresponding increase in the volume occupied by the hydrodynamically equivalent unit in terms of which the volume fraction is computed.

(23) Indeed if the osmotic pressure data are fitted with the value of the second virial coefficient found in light scattering, 3.0 × 10^{-4}, a molecular weight of about 360,000 is obtained, and hence the possibility that the solute is a uniform species does exist.

(24) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(25) For a more detailed consideration of this point see P. Doty, A. H. Holtzer and J. H. Bradbury, THIS JOURNAL, **78**, 947 (1956).

Sedimentation Constant.—Measurements of the sedimentation constant at a number of concentrations are plotted in reciprocal form in Fig. 4. after being corrected to water for both viscosity and buoyancy.[26] From the intercept it is concluded that $S^{\circ}_{20,w}$ is 2.96 ± 0.10 *S*. In the solvent employed the value of s°_{20} is 2.61 *S*.

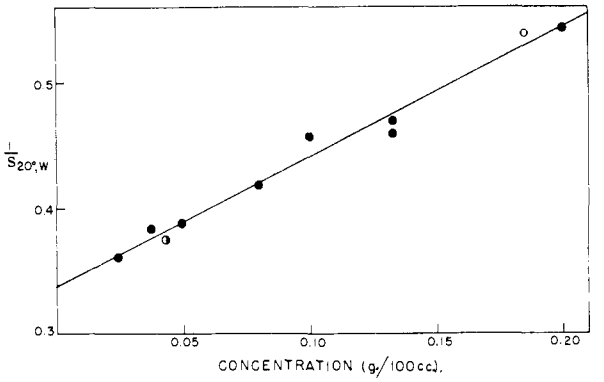

Fig. 4.—Sedimentation of collagen solutions: ●, sample F-1; ○, sample F-3; ◑, sample F-2-1.

If the geometrical form of an ellipsoid of revolution is accepted, then the observed value of s°_{20} can be used in conjunction with Perrin's equation and the intrinsic viscosity to provide a value for the minor axis or the molecular weight. To obtain the length of the minor axis, $2b$, use is made of

$$\frac{s^{\circ}\eta_0\bar{v}}{1-\bar{v}\rho} = (2/9)b^2 \ln 2(a/b)$$

Using the value of 0.700 for $\bar{v}$ and 178 for (a/b), one obtains for $2b$ a value of 14.8 Å. In terms of a cylinder diameter this is 12.1 Å. The molecular weight calculation is readily made from the combined form of Perrin's and Simha's equation.[27] The result is M = 250,000.

The values obtained for $2b$ and M are somewhat smaller than those deduced from other measurements. The differences lie somewhat outside the range permitted by the probable error in the sedimentation constant. It is likely that the origin of the present differences lies in the sensitivity of the quantity $(1 - \bar{v}\rho)$, which occurs in both calculations, to the value assumed for $\bar{v}$. Having been unable to obtain a satisfactory measurement of this quantity for collagen because of the high viscosity of the solutions, we have assumed the value to be the same as that determined for the parent gelatin derived from the collagen, 0.700. However, since it is known that the partial specific volume of some proteins is higher than their denatured products[28] and since a measurement on a different kind of collagen[29] (skin extract with a nitrogen content of only 15% compared to 17.5% for ichthyocol) gives a very high value, 0.78, it is possible that our computed values are systematically low for this reason. A value of 0.75 for $\bar{v}$ would produce agreement with the average of the results obtained from other measurements.

Flow Birefringence.—From the measurement of extinction angle as a function of gradient, one obtains with homogeneous ellipsoids of revolution a result that is interpretable in terms of the length of the major axis provided a rough estimate of the axial ratio is available. From such measurements on several samples at very low concentrations results like those shown in Table II have been obtained. The values of the length are somewhat dependent on the gradient but lie within the range of 2500 to 3000 Å. In view of experience gained in a study of the relation of light scattering to flow birefringence determinations of length in polypeptides in the helical form,[30] we would expect the value of about 2900 Å. to be comparable with light scattering determinations. Instead they differ by about 7%.

TABLE II

EXTINCTION ANGLE AND COMPUTED LENGTHS OF COLLAGEN MOLECULES AS A FUNCTION OF GRADIENT AT 0.025 G./100 CC. CONCENTRATION (SAMPLE F-1)

Angle, deg.	Gradient, sec.$^{-1}$	Length (2*a*), Å.
(45)	0	(2950)
38.4	1325	2880
37.8	1325	2820
37.8	1500	2760
35.7	2130	2710
35.0	2290	2730
32.2	2870	2780
30.4	3930	2660
29.0	4240	2700
28.1	5540	2540

Typical measurements of the birefringence of flow are shown in Figure 5 where $\Delta n/c$ is plotted against gradient. The limiting behavior is reached in the vicinity of 0.08 g./100 cc., and it is noted that the birefringence levels off in a manner typical of rigid rods. The optical factor, $g_1 - g_2$, is found from the data to be about 0.0024. When this is combined with the specific refractive index increment and the partial specific volume in the usual

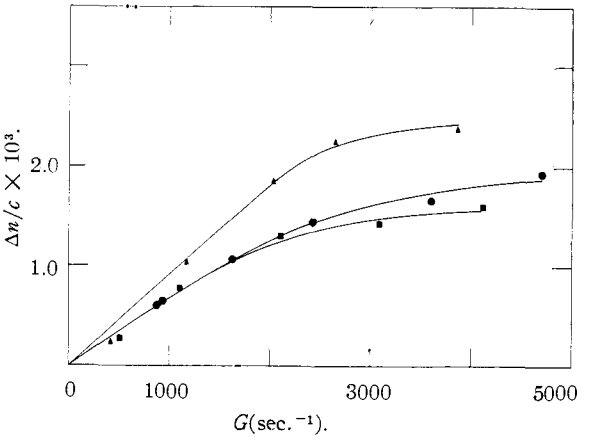

Fig. 5.—Birefringence of collagen solutions: sample F-1, citrate buffer, *p*H 3.7, 20°: △, 0.20 g./100 cc.; ●, 0.12 g./100 cc.; ■, 0.08 g./100 cc.

(26) The 0.15 *M* citrate buffer used as solvent had at 20° a viscosity of 0.01088 poise and a density of 1.015.

(27) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953), A value of 3.42 was used for the parameter β.

(28) K. Linderstrom-Lang, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 117 (1950).

(29) H. Noda, *Biochim. Biophys. Acta*, **17**, 92 (1955).

(30) J. T. Yang and P. Doty, unpublished results.

type of calculation,[31] it is found that the intrinsic birefringence ratio, n_1/n_2, is about 0.998. This lies within probable error of unity; hence, it can only be said that the particle is nearly isotropic but may be slightly negatively birefringent. The easily observable birefringence of flow is therefore nearly all due to form birefringence.

The results obtained from flow birefringence are seen to confirm the rod-like form and the length of the collagen molecule previously deduced from light scattering and from the combination of viscosity and the light scattering molecular weight. In addition the narrow molecular weight distribution indicated by the approximate equality of the osmotic pressure and light scattering molecular weights is also confirmed by the very small range of length detectable over a 5000 sec.$^{-1}$ variation in gradient.

Summary and Discussion.—Although a discussion of the structure of the collagen molecule and its relation to the collagen fibril is taken up in the final section, it is useful to present our results here in tabular form (Table III) and to compare these with other measurements.

TABLE III

SUMMARY OF MOLECULAR CONSTANTS OF ICHTHYOCOL COLLAGEN[a]

Method	Mol. wt.	Length, Å.	Diameter, Å.
Osmotic pressure (no. av.)	310,000		
Light scattering (wt. av.)	345,000	3100	12.8
			(13.3)
Intrinsic viscosity and mol. wt.		2970	13.6
Sedimentation and viscosity	250,000		12.0
	(340,000)		(13.6)
Flow birefringence and viscosity	350,000	2900	13.3
			(13.8)

[a] The values in parentheses are those obtained if $\bar{v}$ is taken as 0.75 instead of 0.70.

From these results we conclude that collagen molecules from this source are rod-like in shape with weight average molecular weight and length of 345,000 and 3,000 Å., respectively, and with a diameter of 13.6 Å. Since these are the first measurements of osmotic pressure and flow birefringence, no comparison with other work is possible. The much higher values found by light scattering by other investigators we have already attributed to unusual difficulties in optical clarification; this is dealt with in Appendix I. The measured values of the sedimentation constant and the intrinsic viscosity agree within probable error with other measurements on ichthyocol.[13] However, the intrinsic viscosity is significantly lower than two reported measurements on tendon collagen[12,29] indicating that the axial ratio for the latter may be about 15% more.

The investigation of rat tail tendon collagen by Noda[29] approaches most closely the results reported here. His study consisted of measurements of sedimentation constant, intrinsic viscosity and diffusion constant. Appropriately disregarding the latter he concluded that if the molecule were rod shaped it would have a molecular weight of 700,000 and a length of 5,000 Å. Our own analysis of his data suggests that these values should be substantially reduced. However, it is unlikely that they could be as low as our results for ichthyocol. Consequently it appears that either the collagen molecule in rat tail tendon is larger or the polydispersity is greater than found in this study. Judging only from intrinsic viscosity determinations,[12] the average dimensions of collagen extracted from skin falls midway between the results for collagen from ichthyocol and rat tail tendon.[32] In conclusion, a sifting of the limited evidence available on collagens other than ichthyocol strongly suggests that they closely resemble the long, rod-like structure established in this study. It is quite possible that the only variations are minor ones in length and amino acid residue composition.

The Denaturation of Collagen

If the loss of the specific structure of a macromolecule without chemical degradation is taken as the meaning of denaturation, then it is evident from the work of Gallop[13] and Cohen[33] that ichthyocol collagen in solution undergoes denaturation in the vicinity of 30° because they observed that the intrinsic viscosity changes from 13.2 to 0.34 and the specific rotation from −330° to −110° after brief exposure to this or higher temperatures. Since reconstitution of collagen fibrils from these heated solutions is no longer possible, the biological function associated with the collagen molecules appears to have been simultaneously destroyed.

Preparatory to a reinvestigation of the properties of denatured collagen, we examined the denaturation itself in somewhat more detail. The reduced specific viscosities of aliquots of three different concentrations of stock solution were determined immediately following a 30-minute exposure to a number of different temperatures. The viscosity measurements were made at the temperature at which the heating had taken place in order to avoid complications due to aggregation (see below). The intrinsic viscosities determined from extrapolation are shown relative to the value for the original collagen solution at 15° in Fig. 6. It is evident that 30 minutes at 23° is sufficient to lower the viscosity a noticeable amount and that in the same time at 33° the denaturation is essentially complete.

If the approximation is made that the denaturation reaction involves only initial and final states, the data of Figure 6 can be fitted with the Arrhenius equation. The best result is found with a value of 81 kcal. for the energy of activation. The full curve Fig. 6 is drawn with this value of the activation energy. In terms of the transition state theory, a value of 230 entropy units is found for the entropy of activation. This high value is comparable with that found for other cases of protein denaturation, and it is indicative of the onset of very considerable rotational freedom in the transition state.

There is a pronounced tendency for the denatura-

(31) See, *e.g.*, R. Cerf and H. A. Scheraga, *Chem. Rev.*, **51**, 185 (1952) and Fig. 19 in particular.

(32) Dr. Gallop has kindly pointed out that his determinations of the intrinsic viscosity for soluble collagen from calf skin are the same as those for ichthyocol, *i.e.*, ref. 12, 13.

(33) C. Cohen, *J. Biochem. Biophys. Cytology*, **1**, 203 (1956).

tion to be reversible. Upon returning the solutions to lower temperatures the optical rotation[33] and viscosity return in the direction of their original values. Light scattering observations show, however, that this is not a reconstitution of collagen molecules but rather a general aggregation, the extent depending on the concentration, temperature and time. This behavior is similar to but more pronounced than that previously studied in solutions of ordinary gelatin.[22] It is evident that the polypeptide strand or strands making up the original collagen molecule have a considerable but incomplete capacity to return to states of intra-chain association characteristic of the native state. As a consequence of this partial reversibility of the denaturation, the entire temperature range in which denaturation occurs must be avoided in studies of collagen or its denatured form. In the 0.15 *M* citrate buffer this temperature range is bounded by approximately 20 and 36°.

It has become common to consider protein denaturation as having the qualities of a phase transition or a coöperative phenomenon. As a consequence the narrow temperature range in which denaturation occurs can generally be lowered by changing the nature of the solvent so as to provide stronger solvation in the denatured state. The lowering of the denaturation temperature range of proteins by the addition of urea is well known. In the case of collagen the role of potassium thiocyanate might be considered comparable since it prevents the gelation of ordinary gelatin. Upon investigation it was found that KCNS did indeed lower the denaturation temperature range so that at concentrations of 2 *M* it was below cold room temperatures (4°). Indeed 2 *M* KCNS dissolves precipitated collagen at neutral or alkaline *p*H but not at acid *p*H. Consequently, 2 *M* KCNS is a solvent for denatured collagen in which aggregation does not occur. This behavior was widely exploited in the studies described in the following section.

Before continuing it is perhaps of interest to draw attention to the similarity between the denaturation of soluble collagen and the thermal shrinkage of collagen.[34] Collagen from a great variety of sources has been shown to undergo an abrupt contraction within a very narrow temperature range when gradually heated in the presence of water or aqueous solution. The temperature of this occurrence depends upon the source of the collagen and the nature of the solute that may be in the aqueous phase. For example, in water or dilute neutral salts this temperature is found to be 60–64° for mammalian skin collagens. The treatment of this shrinkage as a rate phenomenon leads to energy of activations near 140 kcal. for tendon collagen. Moreover, in high concentrations of KCNS the shrinkage takes place at room temperature or below. From thermodynamic considerations Weir and Carter[35] conclude that the only bonds involved in thermal shrinkage are the hydrogen bonds existing between laterally adjacent chains. The similarity of all these observations at the macroscopic level to those on denaturation at the molecular level are obvious. When the collagen molecule is removed from its crystalline environment in the fibril, its stability is lowered by the loss of the crystal energy, and the entropy change upon denaturation is undoubtedly increased. As a consequence the transition temperature at which it melts out into the parent gelatin, being determined by the ratio of the heat content change to the entropy change, is lowered to about 30°. The energy of activation also appears to be somewhat lower for the individual molecule, but this difference may be due to the use of different features (viscosity and linear dimension) as measures of the extent of reaction. In addition the existence of a small but variable amount of cross-linking in the active collagen structures will introduce some differences. However, the coöperative nature of the "melting" out of linear arrays of hydrogen bonds remains as the common feature of both the denaturation of soluble collagen and the hydro-thermal contraction of collagenous tissue.

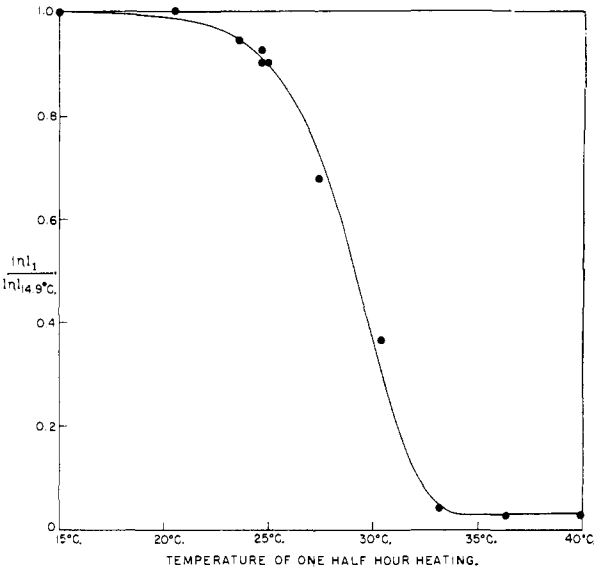

Fig. 6.—Collagen–gelatin transition, as seen in temperature dependence of the intrinsic viscosity: sample F-4, citrate buffer, *p*H 3.7: ●, experimental points; —, calculated assuming $\Delta H^* = 81$ kcal., $\Delta^* S = 230$ e.u.

The Denatured State of Soluble Collagen

Preliminary Investigation and Thermal Lability.—The results of the foregoing section support the finding of Gallop[13] that the intrinsic viscosity of collagen drops to about 4% of its original value upon thermal denaturation. This drastic change signifies a collapse of the original structure which may be accompanied by a substantial drop in molecular weight as well, provided that non-chemically bonded polypeptide strands had made up the native molecule. Previous investigations[10,13] of this denatured form, called parent gelatin by Gallop,[13] had reported it to be monodisperse and of 70,000 molecular weight. If this were true, it would indicate that the collagen molecule were

(34) See K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic Press, New York, N. Y., 1956, Chapter 9, for a perceptive presentation of the important results of the numerous investigations of thermal contraction.

(35) C. E. Weir and J. Carter, *J. Research Natl. Bur. Standards*, **44**, 599 (1950).

made up of five such basic units. Because of the light that such an answer may throw upon the structure of the collagen molecule, we attempted to check it.

In the first experiments use was made of the conclusion from the previous section, that an exposure to 36.5° for 30 minutes would completely transform the collagen to parent gelatin. Light scattering measurements were made on a 0.03% solution of collagen at 20° before and after this heat treatment. By extrapolating the angular measurements to $\theta = 0$ and making the correction for concentration dependence by subtracting $2Bc$ with $B = 3 \times 10^{-4}$, it was found that the molecular weight had changed from 340,000 to 135,000. This drop to 42% instead of 20% of the original value as expected prompted further investigation.

First it was necessary to demonstrate that the denaturation had gone to completion. Longer exposure at 36.5° did show a continued but very slow fall in scattering. Thus we suspected that the denatured state may be somewhat unstable at this temperature. Measurements of sedimentation constant, osmotic pressure and viscosity (see Fig. 7) all confirmed the continued decrease in molecular weight with time. These time dependent changes were not altered when toluene or merthiolate was added. This observation together with the reproducibility of the molecule weight decay indicated that bacterial contamination was not responsible. The possibility that this change was due to a true hydrolysis of particularly labile peptide bonds was supported by observing that the rates of hydrolysis determined by measuring the specific viscosity at 36.5, 55.5 and 73.3° were consistent with a single activation energy, 24 kcal. Since this value was the same as that found by Pouradier[36] for the hydrolysis of commercial gelatin, we accepted this reaction as the explanation of the observed molecular weight decay.

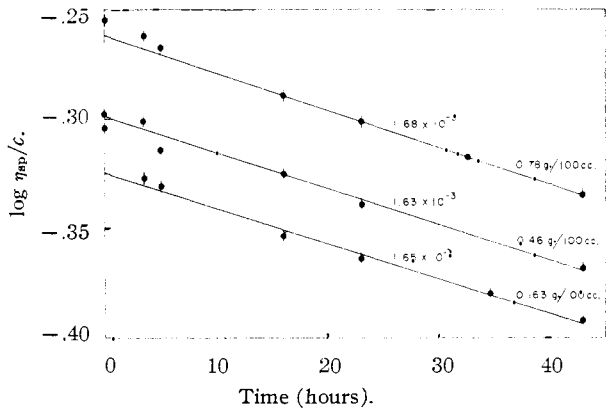

Fig. 7.—Rate of fall of the reduced specific viscosity of gelatin solutions at 36.5°: sample H-1, citrate buffer, *p*H 3.7.

This observation clearly indicated that light scattering, viscosity and sedimentation measurements would have to made immediately following the heating. Moreover, if measurements were made above about 0.1% the temperature must be maintained near 36.5° in order to prevent the reaggregation of gelatin molecules. Although it appeared possible to overcome the difficulties caused by the overlapping of the region of thermal degradation and the region of aggregation by prompt execution of these particular measurements, no such remedy appeared possible for osmotic pressure. It was this type of measurement, providing as it does a number average molecular weight, that was most desirable because it gives, in combination with the molecular weight of the collagen molecule, the number of parent gelatin molecules derived from one collagen molecule.

It was in this context that we explored the properties of 2 *M* KCNS as a solvent for parent gelatin as discussed in the previous section. A parallel investigation of parent gelatin was then undertaken in the two solvents, citrate buffer (0.10 *M* citric acid and 0.05 *M* sodium citrate) of *p*H 3.7 and 2 *M* KCNS at *p*H 7. It was felt that this double effort was necessary because of some uncertainty due to aggregation in citrate buffer and to possible binding of thiocyanate ion in 2 *M* KCNS solutions.

Throughout this study it was essential to ensure that the denaturation had been carried to completion on each sample and that the product of thermal denaturation was the same as that obtained by the addition of KCNS to make a solution of 2 molar. The measurement of the specific rotation was employed as a routine check on the completion of the denaturation since a value of $-100 \pm 10°$ indicated, in agreement with Cohen, that the final state had been reached. The equivalence of denaturation by KCNS and by heating to 36.5° was shown as follows. Concentrated KCNS was added to a solution of collagen in citrate buffer both before and after the latter had been heated at 36.5°. In a second operation the solutions of parent gelatin in 2 *M* KCNS were exhaustively dialyzed against citrate buffer. In both cases the intrinsic viscosity and light scattering molecular weight were found to be the same.

Light Scattering.—The angular intensity distribution of light scattered from parent gelatin solutions was quite small ($R_{45}/R_{135} = 1.13 \pm 0.04$). Typical results corrected to $\theta = 0$ are shown in Fig. 8 for the two solvents. The value in 2 *M* KCNS, 143,000, is in good agreement with these obtained in citrate buffer (146,000 and 148,000),

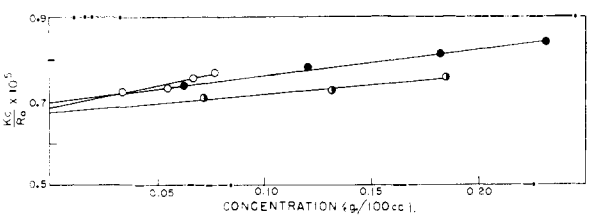

Fig. 8.—Light scattering of parent gelatin solutions: ○, sample H-1 in citrate buffer at 25°; solutions measured immediately after heating to 36.5° for 10 minutes; ◑, sample H-4 in citrate buffer, at 25°; solutions prepared by denaturating collagen by means of 2 *M* KCNS at neutral *p*H, 4° and then removing KCNS by exhaustive dialysis; ●, Sample H-3 in 2 *M* KCNS, *p*H 7, 25°.

(36) J. Pouradier and A. M. Venet, *J. chim. phys.*, **49**, 238 (1952).

The finding that the molecular weight in KCNS is, if anything, below that in citrate indicates that thiocyanate binding has a negligible effect on the light scattering molecular weight. The possibility remains, however, that the collagen solution from which the parent gelatin was prepared contained a small amount of non-dissociable aggregates (see Appendix I) which would tend to make these results high. Therefore a decision on the best value of the light scattering molecular weight is postponed until the later section in which special heating experiments are described.

The angular dependence of the scattering is so small that nothing can be concluded concerning the shape of the parent gelatin molecule and the radius of gyration can only be approximately obtained. If we assume that these molecules are random coils,[22] the root-mean-square end-to-end length, $(\overline{r^2})^{1/2}$, is found to be 530 and 510 Å., respectively, in 2 M KCNS and in citrate buffer. These values may be subject to as much as 25% error, however.

Osmotic Pressure.—The data obtained in 2 M KCNS at 25° are shown in Fig. 9. From the intercept a value of 125,000 ± 10,000 is obtained for the number average molecular weight. This value is unaffected by possible binding of thiocyanate ions in contrast to the light scattering observations. It therefore sets a lower limit on the molecular weight that is considerably above the previously reported value of 70,000.

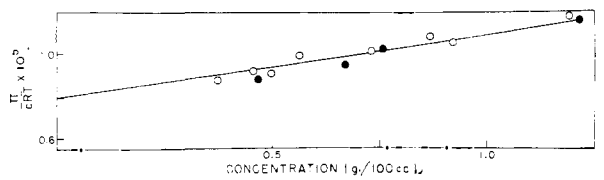

Fig. 9.—Osmotic pressure of parent gelatin solutions: sample H-3 in 2 M KCNS, pH 7, 25°: ●, osmometer 1; ○, osmometer 2.

The second virial coefficient, B, is found to be 3.0×10^{-4} mole cc./g.^{2}. This value is the same as that found in light scattering.

Measurements were also made in citrate buffer at 36.5° by extrapolating the continously decreasing values back to zero time. The results were less accurate but within the larger probable error were consistent with the result in 2 M KCNS.

Sedimentation and Viscosity.—Sedimentation results are shown in Fig. 10. These points have been corrected with respect to buoyancy and viscosity to the conditions prevailing in water at 20°. The result is a value of 3.48 S in 2 M KCNS and 3.77 S in citrate buffer. The latter value is substantially higher than Gallop's value of 3.31 S.

The intrinsic viscosity in 2 M KCNS at 25° is 0.545 and in citrate buffer at 36.5° is 0.44. Gallop[13] reports 0.34 for the latter case at 39.8°. It is of interest to note that the intrinsic viscosity of parent gelatin would fall to 0.34 in about 3 days standing at 36.5° (see Fig. 7).

Aside from characterizing the parent gelatin molecules, these data can be employed to make estimates of the size and weight provided the assumption of randomly coiled configurations is made. Substitution in the Flory–Fox equation

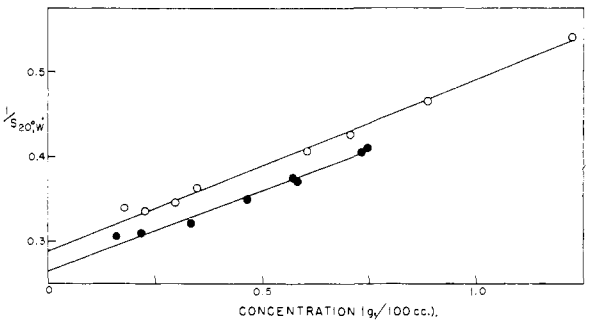

Fig. 10.—Sedimentation of parent gelatin solutions: ○, sample H-3, 2 M KCNS, pH 7; ●, sample H-2, citrate buffer, pH 3.7.

$$[\eta] = \Phi(\overline{r^2})^{3/2}/M$$

leads to values of 335 and 310 Å., respectively, for $(\overline{r^2})^{1/2}$ in 2 M KCNS and in citrate, respectively. These results are considerably lower than those obtained from light scattering. The insensitivity of light scattering in this size range and the difficulty of complete optical clarification prevents one from concluding that this difference is real. The difference is great enough, however, to suggest that the assumption of randomly coiled configurations of *homologous* polymers may not be proper for this system. The measurements of weight and number average molecular weight do not allow an explanation of this difference in terms of polydispersity of molecular weight.

The equation developed by Mandelkern and Flory[37] permits an assessment of the molecular weight from the value of the intrinsic viscosity and the sedimentation constant. The value to be assigned the constant, $\Phi^{1}/^{3}P^{-1}$, appearing in this equation

$$s^{\circ}[\eta]^{1/3}M^{-2/3} = \Phi^{1/2}P^{-1}(1 - \bar{v}\rho)/\eta_0 N$$

lies in the range of 2.3 to 2.7 × 10^6 for the several polymer–solvent systems which have been studied.[38] The value of 2.3 × 10^6 is used here. Since the mean configuration of the parent gelatin molecules may be somewhat temperature dependent, the values of $[\eta]$ and s^0 should be obtained at the same temperature. This was the case for 2 M KCNS solutions but a 12° difference existed in the case of citrate buffer solutions.

The molecular weight was calculated from the foregoing equation using 20° data (for 2 M KCNS, $\eta_0 = 0.915\eta_0^{H_2O}$ and $\rho = 1.092\rho^{H_2O}$; for citrate buffer, $\eta_0 = 1.083\eta_0^{H_2O}$ and $\rho = 1.0167\rho^{H_2O}$ $\bar{v} = 0.705$ for citrate buffer and 0.695 for 2 M KCNS) with the result that a value of 121,000 was obtained in 2 M KCNS and of 128,000 in citrate buffer. A value of 2.1 × 10^6 for $\Phi^{1/3}p^{-1}$ would be required to bring the average of these calculated values up to the weight average value of 140,000 found in light scattering. With the uncertainty due to the value

(37) L. Mandelkern and P. J. Flory, *J. Chem. Phys.*, **20**, 212 (1952).

(38) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press," Ithaca, N. Y., 1953, p. 628. See, however, E. V. Gouinlock, P. J. Flory and H. A. Scheraga, *J. Polymer Sci.*, **16**, 383 (1955).

TABLE IV

SUMMARY OF MOLECULAR WEIGHT MEASUREMENTS OF SOLUBLE COLLAGEN BEFORE AND AFTER HEATING IN CITRATE BUFFER

Sample	Concn., g./100 cc.	Collagen $Kc/R_0 \times 10^6$	Collagen $(Kc/R_0)_{c=0} \times 10^6$	Collagen M	Parent gelatin $Kc/R_0 \times 10^6$	Parent gelatin $(Kc/R_0)_{c=0} \times 10^6$	Parent gelatin M	$\frac{M(\text{collagen})}{M(\text{gelatin})}$
F-2-2	0.068	3.23	2.82	355,000	8.10	7.69	130,000	2.73
H-I	.032	3.10	2.91	340,000	7.60	7.41	135,000	2.52
I-1	.026	3.10	2.94	340,000	7.60	7.44	135,000	2.52
I-1	.027	2.90	2.74	365,000	6.90	6.74	148,000	2.47
				345,000			146,000	2.37

of the constant assessed at about 15% and with an additional uncertainty of 5% in the measurements themselves, the precision of this result is not as great as that of the other measurements. However, it does confirm the values found by osmometry and light scattering and vindicates the rejection of the value previously found.

The Molecular Weight of Parent Gelatin Relative to Soluble Collagen.—The foregoing evidence indicates that the molecular weight of parent gelatin is approximately twice the value of 70,000 previously reported.[9,12] Since this result is not related in any simple way to the molecular weight of 345,000 found for soluble collagen, it seemed important to determine the ratio of the molecular weights with the maximum possible precision. The way to do this seemed to be the following. By carrying out light scattering measurements on a solution at quite low concentration, the value of Kc/R_0 can be determined, and after subtraction of $2Bc$ ($B = 3.0 \times 10^{-4}$), the molecular weight is obtained. This identical solution can then be heated at 36.5° directly in the light scattering cell and a similar procedure applied. Errors in concentration, scattering background and variations in optical clarification are thereby eliminated.

The results of a typical experiment are shown in Fig. 11. (The heating here was carried out at 40° for 5 minutes, since this had been shown to be sufficient to allow the denaturation reaction to go to completion.) After subtracting 0.16×10^{-6} from each intercept and taking reciprocals, the molecular weights of collagen and parent gelatin are found to be 340,000 and 135,000, respectively. The results of similar experiments are given in Table IV. The last entry in this table refers to the independent determinations described in the section on collagen and in the first part of this section. From these results we conclude that the weight average molecular weight of parent gelatin is 138,000 ± 8,000 and that the ratio of the molecular weight of soluble collagen to that of parent gelatin is 2.5 ± 0.15. The values obtained from osmometry fall within the limits of error assigned to this ratio.

The Interpretation of Molecular Weight Data on Parent Gelatin.—The conclusions reached from the experiments just described may be summarized as follows. The number average molecular weight of parent gelatin is 125,000, the weight average is 138,000 and the interpretation of sedimentation and intrinsic viscosity indicates molecular weights in this range. The ratio of the weight average molecular weight of collagen to that of gelatin is 2.5 ± 0.15.

The number average molecular weight of parent gelatin divided into that of collagen should indicate at once the number of molecules into which collagen splits upon denaturation. This gives 2.5 ± 0.6 and hence is capable only of showing that either two or three gelatin molecules are derived from one collagen molecule. The limits of error in osmometry are too great to permit a more definitive answer in this case, although it is likely that further effort in this direction would be successful.

Turning to the weight average molecular weight data, it is apparent that only minimum values can be predicted. That is, if two gelatin molecules are produced, the minimum molecular weight would be 172,500, that is, half the molecular weight of collagen. For three molecules the minimum weight average moleculer weight would be 115,000. It is clear that the measured value of 138,000 ± 8,000 is compatible with three but not with two molecules of gelatin per molecule of collagen. Likewise the ratio of weight average molecular weights, 2.5 ± 0.15 is only compatible with the three molecule possibility. The difference between 138,000 and 115,000 and between 2.5 and 3.0 must be accounted for either in terms of probable experimental error or polydispersity or both.

In order to explain the observed results in terms of three molecules of unequal molecular weight two possibilities exist: either all three molecules are of different molecular weight or two are alike and one is different. For the first possibility, weights of 51,000, 115,000 and 179,000 would yield a weight average of 138,000 and a ratio of 2.5. Of course the full limit of error in the number average molecular weight of 125,000 ± 10,000 would have to be employed to meet the value of 115,000 required of three molecules that must have a total weight of 345,000. For the case of two molecules of equal weight and one of different weight, a similar fit could be obtained for either (1) two molecules of 80,000 and one of 185,000 or (2) one of 43,000 and two of 151,000.

This analysis has been set down in numerical form merely for the purpose of illustrating what kind of polydispersity would have to exist if our results were interpreted literally, that is, as being exact and without error. When the probable errors attached to each determination are taken into account, a vast array of other possibilities arises. However, the limits we have set on our probable errors are such as to exclude the possibility of the chains having equal molecular weight. The rejection of this possibility rests upon its incompatibility with the ratio of 2.5 ± 0.15 between the weight average molecular weight of collagen and parent gelatin. It is therefore important to reassess the uncertainty attached to this ratio. The only significant possibility that appears to have been

overlooked is that arising from compositional differences among the three chains. If, for example, the amino acid residue composition of one chain is different from the other two in such a way that the specific refractive index of this species is substantially higher than the others, its scattering power will be greater. If at the same time this is the chain of greatest molecular weight, the combined effect could result in producing the observed ratio of 2.5 without nearly as much polydispersity as assumed above. Until the equivalence or lack of equivalence of the three chains is established experimentally, it must be kept in mind that the uncertainty of 0.15 attached to the ratio 2.5 by conventional criteria based on the assumption of chains of homologous composition, does not allow for variations in composition of the chains. The consequence of this is that the extent of polydispersity illustrated in the above considerations can be lower not only due to the probable error attached to each of our determinations but also because of the effects that may arise from compositional heterogeneity. If indeed there are differences in composition between the chains, the additional possibility exists of having chains of equal degree of polymerization but of significantly different molecular weight.[39] When these additional possibilities are taken into account, it is clear that no reliable estimate of the possible polydispersity in parent gelatin can be made at present. However, it does appear most unlikely that all the factors mentioned here, working in concert, could account for our observations if the three parent gelatin chains were of uniform degree of polymerization, that is, made of the identical number of residues. The search for possible difference among the molecules of parent gelatin is therefore obviously important.

Discussion

The relation of our observations to closely related investigations of others has already been taken up at the appropriate places in the three preceding sections. In this final section we wish to examine various points of contact between this work and investigations of collagen by electron microscopy and structural studies based on X-ray diffraction.

In seeking to relate the soluble collagen particle we have described to the native form of collagen, reference must be made to terminology presently employed to describe the successive levels of collagen structure.[40] The microscopically visible fiber (primitive fiber) is composed principally of a bundle of fibrils which are readily distinguishable in electron microscopy. These fibrils are considered to be made up of a hypothetical thinnest unit filament, the protofibril, which carries the essential chemical and configurational structure of collagen. It is part of the concept of the protofibril that its continuity extends for very long distances, perhaps as long as the fibril itself, and that at least in native collagen its building units all point in the same direction giving rise to what is known as a polarized structure.

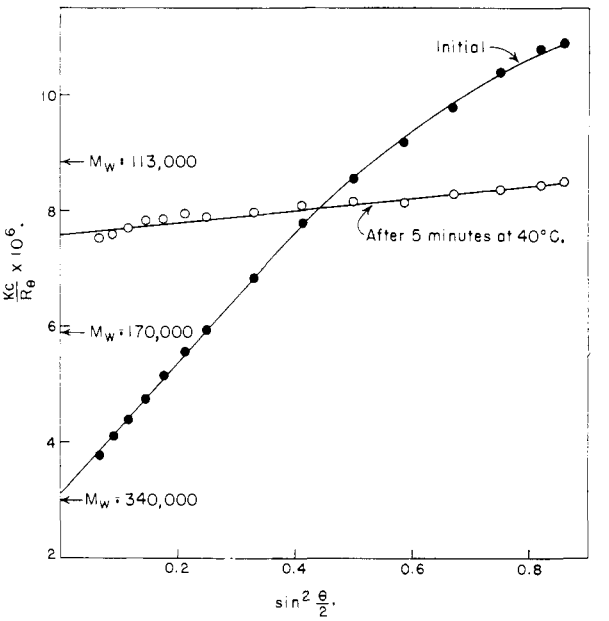

Fig. 11.—Denaturation of collagen solution: sample I-1; citrate buffer, *p*H 3.7: ●, light scattering of 2.6×10^{-4} g./cc. solution before denaturation; ○, light scattering of identical solution after being heated at 40° for five minutes. Arrows indicate the molecular weight of collagen after correcting scattering data to zero concentration, and the expected intercept, if collagen molecule divided into either two or three equal pieces.

The most obvious morphological characteristic of native collagen at the electron microscopic level is the long spaced periodicity which is often quoted as 640 Å. Actually, this is an average value of a fairly wide distribution and recent evidence[41] argues that the mean may be as much as 100 Å. greater. Low angle X-ray diffraction requires a narrower distribution for its occurrence; average values of 640 Å. were originally reported, but more recent measurements indicate somewhat higher values. For the purposes of this discussion 700 Å. will be used.

Previously, when the evidence for a 70,000 molecular weight unit was unchallenged, it was tempting to identify this unit as the part of the protofibril corresponding to the long spacing of 700 Å. Neither the parent gelatin molecules nor the soluble collagen molecule can now be considered as having a dimension in the range of 700 Å. The soluble collagen molecule extends for at least four times this distance, and it seems clear that the structural features which give rise to this periodicity must therefore be repeated several times within the collagen molecule. These structural features may be either compositional or configurational and may in addition determine the relative translational position of nearest neighbor molecules in the fibril. This situation is then analogous to the fact that the long spacing seen in fibrin (230 Å.) is considerably less than the length of fibrinogen (400–500 Å.).

(39) In this connection the suggestion from recent structural work, that one of the chains contains principally proline, hydroxyproline and glycine, does not provide an example of this point because the average residue weight in this case is about the same as that of collagen as a whole. However, such a chain may have a value of dn/dc differing somewhat from the others.

(40) R. S. Bear, "Advances in Protein Chemistry," Vol. VII, Academic Press, New York, N. Y., 1952.

(41) F. O. Schmitt, private communication.

Various proposals for the location of the long spacing within the collagen molecule are taken up below.

As mentioned in the Introduction, the discovery of two new fibrous forms of collagen in precipitates formed under special conditions[14,15,42] had special relevance. The one form, called fibrous long spacing, exhibits symmetrical periodicities of 1800 to 3000 Å. The other form consists of segments having non-symmetrical interbands and total lengths of 1500 to 3000 Å. From these results it was suggested that these new forms, as well as the native form, arose from different types of aggregation of the identical building unit which was called tropocollagen. This unit was thought to have a length compatible with the observed lengths of 1500 to 3000 Å. and to have a small but unspecified cross-section. It seems apparent that the collagen molecule characterized in our studies fits fairly well the higher limit of the specifications of the hypothesized tropocollagen particle. If all three forms can be prepared from samples of soluble collagen having the characteristics given here, it appears that this term may well be applied to what we have heretofore called soluble collagen. The origin of the wide distribution of lengths thus far observed for the periods in the fibrous long spacing and the over-all lengths of the segment long spacing form is not yet established, and it is obvious that considerable work remains in quantitatively relating what we have described as soluble collagen to the three solid state forms.

The deduction of the mutual arrangement of polypeptide chains in collagen fibrils from X-ray structural studies has been the aim of a number of workers[30,43] and appears now to be reaching fruition in the refinement that has been given to the coiled-coil structure of Ramachandran and Kartha[44] by Rich and Crick[45] and Cowan, McGavin and North.[46] This structure consists of three polypeptide chains each having approximately a three-fold screw axis. The structure is maintained by hydrogen bonds which unite residues on about the same axial level, but the proposals differ with respect to the detailed disposition of the hydrogen bonds. The degree to which the Rich–Crick structure meets the requirements set by Bear[47] offers very strong support to the contention that this structure corresponds to the principal configurational features at least in the crystalline regions of the fibril.

Our conclusion that denaturation produces three molecules from the original collagen molecule is consistent with the X-ray structure and the identification of the three molecules with the three strands would be assured if the mass-to-length ratio for the collagen molecule and its diameter are compatible with the X-ray structure.

The mass-to-length ratio, M/L, is given in the last column of Table I for several light scattering measurements. From these the value is seen to be 110 ± 4 avograms/Å. This is to be compared with the value of 98[40] required by X-ray diffraction. The high angle X-ray diagram shows the nearly hexagonally packed rods to be 12 Å. apart in dry material and this separation increases somewhat with hydration. Our measurements lead to a value of 13.6 Å. for the diameter in solution where hydration would be complete. Thus on these two points we find good agreement between the collagen molecule as characterized in solution and the requirements of the X-ray structure of the collagen fibril.

After this successful comparison the only point left for discussion here is the fitting together of the ends of the soluble collagen molecules in the fibril. Since the way in which this is accomplished has not been elucidated, some very tentative suggestions arising from our work may not be out of place. It may well be that the collagen molecules are of uniform length and cross-section and that the strength of the fibril derives from the overlapping of the molecules as they are laid down in fibril formation. The existence of the 700 Å. spacing strongly suggests, however, that the adjacent molecules must be in register at least with respect to this period. But since there appear to be four such periods in each molecule, three different positions of relative linear displacement would still be permitted, and thus cleavage planes perpendicular to the fibril would be avoided.

However, the basis of this view, that collagen molecules are of both uniform length and cross-section, is not compatible with our tentative conclusion that there is some significant degree of polydispersity among the molecules of parent gelatin and hence among the three chains making up the collagen molecule. If this possibility of unequal chain length is accepted, several interesting conclusions follow.

If the three chains are of unequal length but held together so that a basic repeat distance of about 700 Å. is preserved, the end-to-end addition of collagen molecules in such a way as to maintain three chains at all points is only possible for an arrangement that has a single chain protruding at one end and two chains at the other. The various possibilities are shown diagrammatically[48] in the upper part of Fig. 12. The straight portions of the lines indicate single polypeptide chains in regions where one or two neighbors exist and in which rigidity would be expected. The coiled part of the end of a line indicates a single polypeptide chain extending from a group of two or three. This part would be randomly coiled and flexible. The dots on the lines correspond to the normal spacing of 700 Å. and as a consequence the length of the rigid portions of all the possibilities shown would be 2800 Å. or four times the normal spacing. A dangling, flexible chain at one end would increase the length to about 3000 Å. as is observed and would in addition account for the observed value of M/L

(42) F. O. Schmitt, J. Gross and J. H. Highberger, in "Fibrous Proteins and Their Biological Significance," Academic Press, New York, N. Y., 1955, p. 148.

(43) J. Kendrew, in "The Proteins," Academic Press, New York, N. Y., 1954.

(44) G. N. Ramachandran and G. Kartha, *Nature*, **176**, 593 (1955).

(45) A. Rich and F. H. C. Crick, *ibid.*, **176**, 915 (1955).

(46) P. M. Cowan, S. McGavin and A. C. T. North, *ibid.*, **176**, 1062 (1955).

(47) R. S. Bear, *J. Biochem. Biophys. Cytology*, in press.

(48) In the actual molecule, the three chains are related at any axial point as the three corners of an equilateral triangle and as such do not possess the non-equivalent position is shown in a two-dimensional representation.

being 10% larger than that required by X-ray diffraction.

The ratio of the weight to number average degree of polymerization for the two possibilities shown at the top of Fig. 12 is 1.20. This ratio fits the observations and corresponds to the case of three unequal chains discussed previously in "the Interpretation of Molecular Weight Data on Parent Gelatin." The ratio is much less, 1.04, for the next pair of possibilities.

There are two very slight indications in our work that are in favor of a dangling, coiled, single chain protruding from the rigid portion of the molecule. One is the fact that the length determined from light scattering was found to be consistently somewhat longer than that obtained from flow birefringence. The difference is small but from our experience with similar comparisons in other rod-like systems (synthetic polypeptides in the α-helical configuration[30] and in tobacco mosaic virus), it would be expected to lie in the opposite direction. The presence of a coiled chain would increase the light scattering value much more than the flow birefringence value and hence lead to the observed result. The other observation concerns the fitting of the angular scattering data with the theoretical envelopes. When this is done in the form of the reciprocal plot (Fig. 2), it is found that the high angle points consistently fall above the theoretical plot for monodisperse rods. Polydispersity in a sample of rod-like molecules could only cause the opposite effect, however; that is, the high angle data would fall below the theoretical curve for monodisperse rods. Here again this small discrepancy could be accounted for if indeed an effective increase in scattering material occurred at one or both ends.

For completeness the possibilities resulting from the staggering of chains of equal length are shown in the lower part of Fig. 12. These possibilities would also have protruding, randomly coiled, single chains at the ends like the examples just discussed. They do not, however, satisfy the indications we have of polydispersity. The double-staggered possibility has already been considered by F. O. Schmitt and others[41] with regard to explaining electron microscopically observed periodicities.

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Appendix I

Since the major conclusions of this investigation rest on light scattering measurements and since it was this type of measurement which led to earlier reports of much higher molecular weights for collagen, as well as lower molecular weights for parent gelatin, additional details of this part of our study are presented here.

In the first part of this investigation apparent molecular weights of collagen were obtained that were much higher than those finally observed. Some of these early results are shown in Table V.

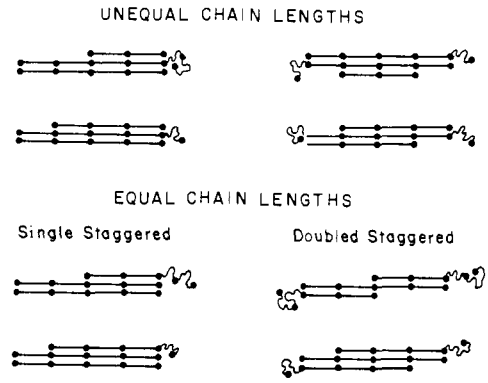

Fig. 12.—Possible arrangements of polypeptide strands in the collagen molecule.

Although the values range from 1.5 to 8 times the value finally established for soluble collagen the intrinsic viscosities are the same. Likewise sedimentation constants and the flow birefringence behavior for samples BC and F-2-1 were found to be the same as the values reported earlier in this paper. These observations show that the hydrodynamic characteristics of the solute are constant while the light scattering molecular weight and length have varied. This contradiction could only be resolved by assuming that a small amount of material of considerably larger size than the actual collagen molecule was present in varying amounts. Since the molecular weight and length determined by light scattering depend on highly weighted averages and since the hydrodynamic methods may not reflect any contribution of minor components having different properties, this possibility seemed likely.

TABLE V

RESULTS OBTAINED ON COLLAGEN SOLUTIONS BEFORE PROPER OPTICAL CLARIFICATION

Sample	Apparent mol. wt.	Apparent length, A.	Intrinsic viscosity
BC	2,300,000	8150	10.0
E-2	830,000	5500	11.4
F-1	512,000	4400	13.4
F-1-r	570,000	4650	12.0
F-2	500,000	4300	12.4
F-2-1	580,000	4750	10.5
F-3	465,000	4400	11.8

As a consequence we undertook a reinvestigation of the light scattering determinations and found that the apparent molecular weight depended strongly on the concentration of the stock solution which was ultracentrifuged for the purpose of clarifying the solution prior to its being added to solvent in the light scattering cell. When the concentration of the stock solution was below 0.2 g./dl., it was found that ultracentrifugation could lower the apparent molecular weight to the limiting value of 345,000 ± 30,000 reported in this paper.

In some solutions the ultimate value was found after a single centrifuging of about 1-hour duration; in other cases three and even four successive centrifugations were required. Concentrations were measured after centrifuging unless the concentration was too low (<0.06 g./dl.) for precise concentration determination.

In Table VI and Fig. 13 we have collected data for sample I-1 in order to illustrate the nature of

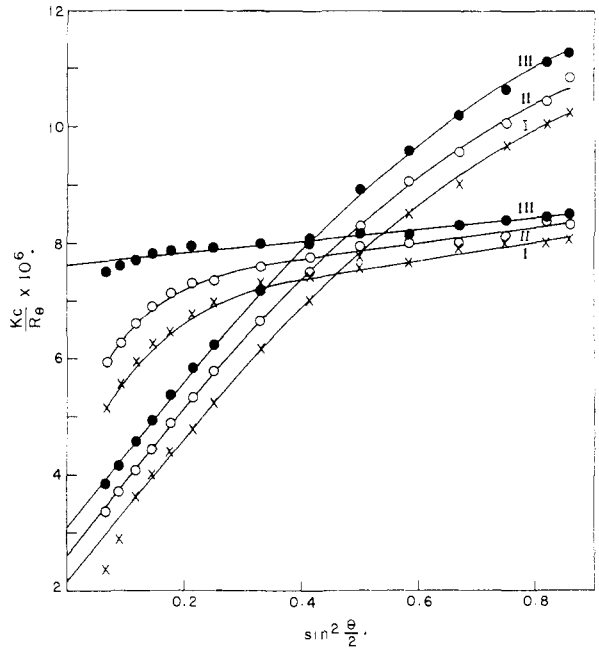

Fig. 13.—Effect of successive centrifugations on light scattering from collagen and gelatin solutions: sample I-1, citrate buffer, *p*H 3.7. Numerals I, II and III refer to entries 1, 2 and 4 of Table VI.

this problem. After each centrifuging a portion of the solution was added to solvent in the light scattering cell in order to produce a concentration near 0.02 g./dl. and the reciprocal scattering envelope obtained before and after heating are recorded in Fig. 13. The molecular weights were obtained after correction for $2Bc$.

TABLE VI

LIGHT SCATTERING RESULTS OBTAINED AFTER REPEATED CENTRIFUGATIONS

Concn., g./dl.	Centrifuging time and speed[a]	Apparent mol. wt. of collagen	Apparent mol. wt. of gelatin	$\frac{M(\text{Collagen})}{M(\text{Gelatin})}$
0.11	90′ at 19,000	490,000	152,000	3.22
.11	60′ at 19,000	415.000	144.000	2.88
.073	120′ at 29,000	365,000	148,000	2.46
.056	600′ at 17,000	340,000	135,000	2.52

[a] Rotor No. 21 of Model L Spinco Ultracentrifuge used except in third entry where No. 30 was used.

The stepwise removal of highly scattering contaminant is observed not only through the lowering of the molecular weights but also in the character of the reciprocal scattering envelope shown in Fig. 13, where the disappearance of downward curvature in the parent gelatin curves at low angles is the certain indication of the attainment of ultimate clarification. From the successive values of the ratio of the molecular weights of collagen and parent gelatin, it can be seen that value of 3 can be obtained in a partially clarified solution but that upon completion of the clarification the ratio reaches the value of 2.5. Further ultracentrifugation does not lower the results below this figure.

Although no attempt was made to identify the nature of the optical contaminant that was removed, it is fairly clear that it was not linear, rod-like aggregates of collagen molecules because these would have shown their presence by raising the intrinsic viscosity and lowering the extinction angle, as well as by causing the length to be at least proportional to the light scattering molecular weight. Moreover their sedimentation rate would have been so close to that of the soluble collagen molecule that separation could not have been obtained. However most other possibilities cannot be excluded. The contaminant could be clusters of collagen molecules, an end-to-end non-linear aggregate of collagen molecules or non-collagenous material.

Appendix II

Long, thin rods possessing an intrinsic anisotropy will cause a depolarization of the scattered light resulting from their shape which will be a function of the scattering angle θ. In this case, both the length and the molecular weight calculated from light scattering data obtained using unpolarized light would be in serious error. To determine whether solutions of soluble collagen exhibited this depolarization, we measured the scattering of a 0.027% solution (sample I-1) using vertically polarized incident radiation. Figure 14

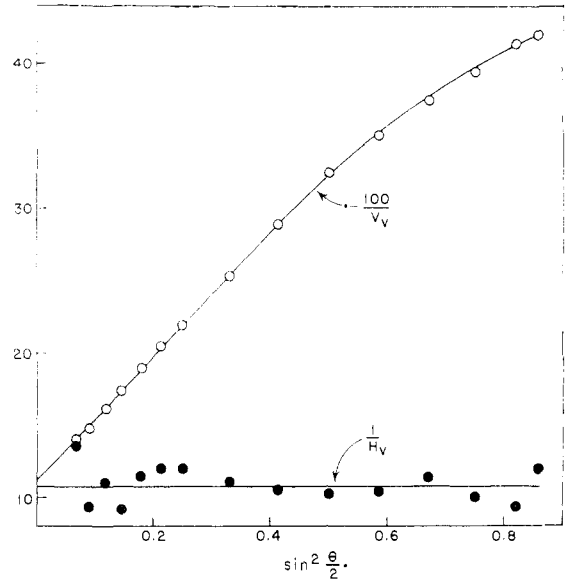

Fig. 14.—Depolarization of light scattered from solution of soluble collagen: sample I-1, 2.7×10^{-4} g./cc., citrate buffer, *p*H 3.7, 20°.

shows the results obtained when the analyzer was parallel (V_V) and perpendicular (H_V) to the incident beam. The depolarization, $\rho_V = H_V/V_V$, at $\theta = 0$ was obtained by extrapolating $100/V_V$, and $1/H_V$ to zero angle. ρ_V was 0.01 and thus the error in the molecular weight of collagen due to such depolarization is well within the probable error of the measurement. The length as determined from the limiting slope of the reciprocal scattering intensity was found to be 3040 Å. using polarized light and 3180 Å. with unpolarized light. As these two values are also within the error of the measurement, we conclude that depolarization corrections are negligible.

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