The Physical Characterization of Monomeric Tropocollagen*

P. F. Davison and M. P. Drake†

ABSTRACT: Monodisperse solutions of calfskin tropocollagen molecules obtained by pronase digestion or sonic irradiation have been studied by physical methods. Several physical parameters, including a partial specific volume of 0.655 and a molecular weight of 260,000 (±10,000), have been determined for the tropocollagen molecule. It is concluded that most soluble tropocollagen solutions contain linear polymers; such polymers are believed to be stages in the formation of insoluble collagen fibers.

Since the important experiments of Boedtker and Doty (1956) which established the high asymmetry and approximate dimensions of native tropocollagen molecules in solution, a number of other physical investigations on these solutions have been reported. One of the factors that has plagued investigators has been the tendency of the acicular molecules to aggregate, particularly under certain conditions of ionic strength and in certain solvents (*e.g.*, Barkin and Oneson, 1961; Rice *et al.*, 1964). In studies in this laboratory over several years, most of the physical properties of different preparations have varied little, but the intrinsic viscosity of successive preparations has increased, due, we now believe, to increasingly effective prevention of proteolytic attack by contaminating proteases and microorganisms and of slow hydrolysis of labile bonds in acid solutions. It has also been noted that the precipitated aggregates of tropocollagen, characterized in the electron microscope as segment-long-spacing aggregates, tend to appear as linear fibrils in most recent preparations, but on storing the solutions these aggregates become more and more of the monomeric type. Recent studies reported in the preceding and earlier papers (Rubin *et al.*, 1963, 1965; Drake *et al.*, 1966) have demonstrated that the viscosity of native tropocollagen solutions can be decreased by the action of proteolytic enzymes, and it was concluded that in an extract of tropocollagen there occur covalently linked polymeric as well as monomeric molecules. The tropocollagen molecules themselves exhibit a pronounced tendency to interact and to aggregate; physical characterization of such solutions containing polymers and aggregates therefore has been difficult. As described in the preceding paper, the polymers can be disrupted by certain proteases, particularly pronase under appropriate conditions, and the resulting solution comprises monomeric tropocollagen shorn of pronase-accessible peptides. Such monomeric, "peptideless" molecules differ in interaction properties from tropocollagen; in particular, they are soluble in most aqueous salt solutions over the whole pH range except between pH 9–11, *i.e.*, close to the isoelectric point. The physically monodisperse character of the solutions makes them suitable for the physical characterization of the protease-resistant triple-helix body of the tropocollagen molecule.

As described in this paper, the polymeric aggregates of tropocollagen molecules can also be disrupted by controlled sonic irradiation of a tropocollagen solution. Such a solution is also suitable for physical examination. The physical characterization of pronase-treated and sonicated tropocollagen solutions is presented in this paper. Preparations containing molecules in this state of dispersion have doubtless been available to earlier investigators who employed solutions sufficiently aged, *i.e.*, in which contaminating proteases have had the opportunity to degrade the polymers. Paradoxically, it would appear that in solutions treated conservatively to minimize degradation, the problems in physical characterization are greater.

Materials and Methods

The acid-soluble calfskin tropocollagen preparation used in these experiments was isolated by methods previously described (Rubin *et al.*, 1965).

Pronase-treated collagen for physical characterization was obtained by digesting tropocollagen in calcium acetate pH 7.3 solutions for a period of 12 hr at 20° as described in the preceding paper (Drake *et al.*, 1966). The solutions were freed of pronase by repeated precipitation of the tropocollagen with KCl as previously described and the appropriate buffers were dialyzed into the redissolved tropocollagen.

Most of the physical techniques, methods, and apparatus also have been previously described. Viscosity was measured in simple Ubbelohde viscometers and also

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† Supported by a U. S. Public Health Fellowship (5F3 AM-11,175) from the National Institute of Arthritis and Metabolic Diseases. Present address: Department of Biochemistry, School of Basic Medical Sciences, The University of Tennessee, Memphis 3, Tenn.

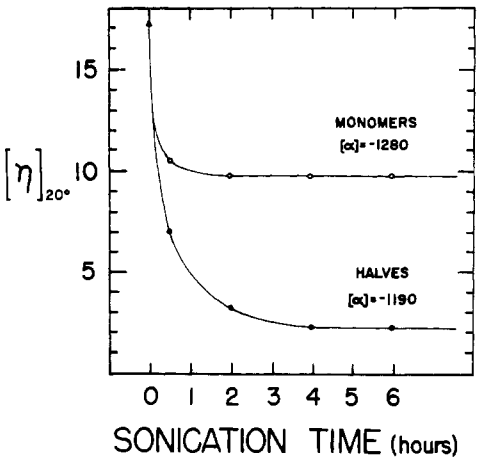

FIGURE 1: The change of intrinsic viscosity of helium-flushed calfskin tropocollagen as a function of sonic irradiation time and of sonic intensity. Step 1 power setting producing monomers, and step 2 producing halves.

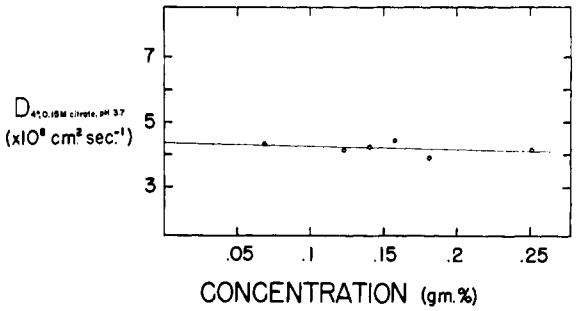

FIGURE 2: Extrapolation to zero concentration of the measured diffusion coefficients, $D_{4°}$ (0.15 M citrate, pH 7), for pronase-treated tropocollagen at 4°. The regression line equation is $y = 0.43_6\ (\pm 0.01) - 0.10_9 x$ ($\times 10^{-7}$ cm²/sec).

in a special four-bulb, low-shear Ubbelohde viscometer. Unless otherwise indicated the solvent used in these experiments was 0.15 M sodium citrate, pH 3.7.

Sonic Irradiation. The solutions were irradiated by a Bronson Sonifier S75, which is an electrostrictive 20-kc apparatus provided with a stepped power control. The solutions were placed in a parabolic-section stainless-steel container which could be screwed to the probe and which was provided with gassing ports for flushing the solution with a suitable gas as well as for irradiating under high or low gas pressures. The minimum volume of liquid used was 3 ml. The liquid container and probe were immersed in ice during sonic irradiation.

Sedimentation Velocity. Measurements of the sedimentation velocity of pronase-treated tropocollagen were made in 30-mm synthetic-boundary Epon cells at 39,500 rpm at 4° in a Spinco Model E centrifuge. The hypersharpening of the boundaries, and the long-path-length cells permitted measurements down to 0.01% concentration. Sedimentation velocity measurements at 20 and 40° were made in 12-mm Epon double-sector cells at 56,100 rpm at concentration between 0.1 and 0.4%.

Sedimentation Equilibrium. Equilibrium centrifugation experiments were conducted over 48–72 hr; the column heights in the Epon double-sector cells were less than 3 mm. The Rayleigh optics of the Spinco ultracentrifuge were carefully adjusted by the methods described by LaBar and Baldwin (1962). The interference photographs were measured on a Gaertner microcomparator and the molecular weights were calculated from plots of ln J against r^2 (Van Holde and Baldwin, 1958).

Diffusion. Diffusion measurements on pronase-treated tropocollagen were made in the Model H Spinco apparatus. The ascending boundaries were displaced into view very slowly (20–40 min) but the displaced boundaries nevertheless rapidly became skewed as solvent adhering to the walls of the cell slowly moved to the boundary position. The boundaries were sharpened by careful aspiration through a fine needle and the needle was left in place for several hours; subsequent resharpening was performed as was deemed necessary. The boundary was photographed after each sharpening operation and, when no asymmetry developed in the course of 2–3 hr, the final resharpening was accomplished, the needle was withdrawn, and the cell was closed. Diffusion was allowed to proceed for between 12 and 14 days, and photographs were taken daily. The Rayleigh interferograms were carefully measured and analyzed by the method of Creeth (1955). The apparent diffusion coefficients (D') calculated from the times elapsed between the making of the boundary and the taking of the photograph were plotted as recommended by Longsworth (1947) and the true diffusion coefficient (D) was obtained by extrapolation.

Electrophoresis. The electrophoretic mobility of pronase-treated tropocollagen and of sonicated native (fraction 2A) tropocollagen was measured in various solvents in a Spinco Model H apparatus. The solutions contained 0.1% protein.

Partial Specific Volume. The partial specific volume of sonicated and pronase-treated tropocollagen solutions was measured in a density gradient (Linderström-Lang and Lanz, 1954). The gradient was formed by a conventional gradient maker (Britten and Roberts, 1960) between mixtures of bromobenzene and kerosene adjusted to suitable densities. The density range across the 30 cm long column was 0.02. The thermostat bath was regulated to 20 ± 0.001° and the positions of the drops were measured to 0.02 mm with a Swift Cathetometer. The density gradient was calibrated with carefully weighed solutions of potassium chloride whose densities were obtained by interpolation from values in the "International Critical Tables." Before and between experiments the columns were saturated with water by dropping an emulsion of 6% aqueous KBr solutions in kerosene into the columns. After each experiment

the columns were cleaned of the suspended drops by dropping in fine washed dry sand.

Melting Curves. The temperature of the sample (in a water-jacketed polarimeter tube) was increased in a series of steps from 25 to 40°, and the specific optical rotation values used to construct the melting curves were the 30-min reading in each step. Each curve was constructed of points made in two or more separate runs; approximately seven temperature values were obtained in the 4 hr required for each run.

Results

Sonic Irradiation. The characteristics of the sonicated solutions at any given power setting on the sonifier depended upon the nature and the pressure of the gas over the solution. A convenient combination was helium gas at atmospheric pressure. Figure 1 shows the viscosity of a tropocollagen solution as a function of the irradiation time at two power settings. In this, as in most other experimental instances, oxygen was excluded to obviate free-radical chemical attack on the tropocollagen.

The results may be presumed to arise from mechanical shearing forces in solution (Gooberman, 1960; Freifelder and Davison, 1962), and the asymptote in Figure 1 represents in each case the limiting degradation induced by the given sonic treatment. Electron microscopic examination of segment-long-spacing aggregates from step 1 sonic samples showed only monomeric segments with no sign of any fragments. The product from a step 2 sonicate showed only fragments. Sedimentation studies on denatured step 1 sonicates showed that no α chains had been ruptured.

Monomeric Tropocollagen Solutions. For the physical characterization of monomeric tropocollagen, the sonicate produced by a 3-hr irradiation at 0° using step 1 under helium gas was used. This material showed quite different aggregation properties when compared to the parent tropocollagen solution but no demonstrable dialyzable products were produced as a result of sonication nor, at these power settings, was there more than a 5% loss of optical rotation in the solution. The latter finding signifies that the pronounced lowering of viscosity cannot be attributed to denaturation of some of the molecules but must be due to a depolymerization of some of the tropocollagen. It is concluded that polymeric end-to-end or partly overlapping aggregates of tropocollagen molecules are broken to monomeric structures by this treatment.

The other monomeric tropocollagen solution used was prepared by a limited pronase digestion (12 hr, 20°) of tropocollagen (Drake *et al.*, 1966). This solution has a similar viscosity to that of sonically irradiated tropocollagen (10 dl/g) and the segment-long-spacing aggregates seen in the electron microscope also represent only monomeric structures.

Physical Characterization of Monomeric Tropocollagen Solutions. Solutions of tropocollagen sonically irradiated, or treated by pronase, are manageable at concentrations up to 1.3%; in contrast, calfskin tropocollagen becomes highly viscous and gellike at concentrations higher than 0.5%. This fact made it feasible to measure the partial specific volume ($\bar{v}$) for native tropocollagen (a figure supplementing the more generally quoted measurement which has been obtained by a number of authors on gelatin) as well as other physical properties at concentrations higher than normally obtainable.

TABLE I: Partial Specific Volume Measurements on Monodisperse Calfskin Tropocollagen.

Tropocollagen	Concn (g %)	$\Delta\rho \times 10^5$ (g/cc)		$\bar{v}$ (ml/g)
Helium-sonicated	0.426	141		0.659
	0.426	145		0.651
	0.426	146		0.649
	0.426	143		0.655
			Av	0.654
Pronase-treated	1.37	447		0.666
	1.37	456		0.654
	1.37	449		0.664
	1.37	450		0.663
	1.37	458		0.658
	0.493	171		0.644
	0.493	167		0.652
	0.493	171		0.644
	0.493	168		0.650
			Av	0.655

Calculation: $\bar{v} = [1 - (\Delta\rho/C)]1/d_s$, where $1/d$ (0.15 M citrate) = 0.986 ml/g.

Partial Specific Volume. Two series of measurements were made separately by the authors using several different batches of calibrating potassium chloride solutions. The values for $\bar{v}$ obtained in this manner are listed in Table I. In each case the concentration of the tropocollagen solution was measured by Kjeldahl nitrogen determination; the content of tropocollagen was taken as 17.6% (Rubin *et al.*, 1965). In our hands the nitrogen determinations are precise to ±1% and a somewhat smaller error could be assigned to the density difference measurements; these errors therefore limit the accuracy with which $\bar{v}$ can be given. The mean value for $\bar{v}$ was found to be $0.65_5 \pm 0.010$ for both pronase-treated and sonicated tropocollagen.

Diffusion Measurements. A series of diffusion measurements was made on pronase-treated tropocollagen. A value for the diffusion coefficient extrapolated to zero concentration was found to be 0.43_6 (± 0.01) $\times 10^{-7}$ cm^2 sec^{-1} (Figure 2).

Sedimentation Velocity. Since diffusion measurements were made at 4°, sedimentation velocity measurements for molecular weight calculations were made at the same temperature. The sedimentation velocity measure-

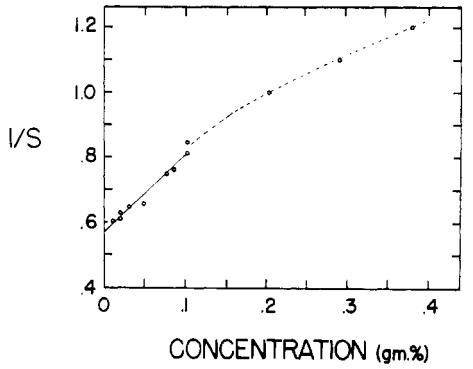

FIGURE 3: Extrapolation to zero concentration of the reciprocal of the measured sedimentation coefficients for pronase-treated tropocollagen at 4°. Only values for concentrations below 0.1% were used for calculating the regression line. $y = 0.57_3\ (\pm 0.016) + 2.22x$ S^{-1}.

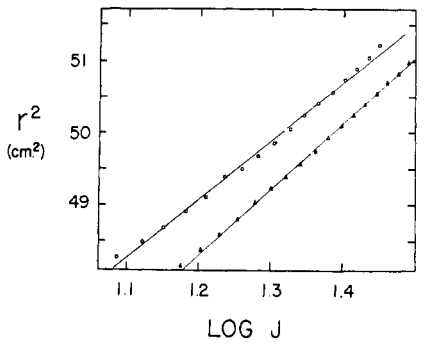

FIGURE 4: Typical plots of the logarithm of the fringe number J (*i.e.*, concentration) against the square of the fringe position radius for equilibrium molecular weight measurements. O, Pronase-treated tropocollagen (0.485%); Δ, sonicated tropocollagen (0.526%).

ments on a series of solutions of pronase-treated tropocollagen are shown in Figure 3; the extrapolated sedimentation coefficient at 4° is 1.75 (±0.05) S. From the above determined sedimentation and diffusion coefficients and a mean partial specific volume of 0.65_5 (albeit the value measured at 20°) the molecular weight of pronase-treated tropocollagen was calculated to be 268,000 using the equation of Svedberg and Pederson (1940). The sedimentation coefficients corrected to standard conditions may be compared for runs made at 4 and 20°; at 4°, $s^0_{20,w}$ is calculated to be 3.8 S, while at 20°, it is 3.4 S. The runs at 20°, however, were not made to the same low concentrations as the 4° series, and if a similar curvature to that in Figure 3 occurred, this figure would be too low.

Sedimentation Equilibrium. A series of experiments with two different preparations of pronase-treated tropocollagen and one preparation of helium-sonicated tropocollagen were made by equilibrium sedimentation. Two typical plots of the concentration distribution in the cells are shown in Figure 4. A marked departure from linearity in the pronase-treated samples was noted; this nonideal behavior probably reflects the large concentration-dependent behavior shown in Figure 3. Although the concentration dependence of the apparent equilibrium molecular weights differs markedly between pronase-treated and sonicated tropocollagen (Figure 5), the extrapolated figures are similar, corresponding to molecular weights of 2.5 (±0.5) × 10^5 and 2.6 (±0.3) × 10^5, respectively.

Intrinsic Viscosity. The intrinsic viscosities of several sonicated and pronase-treated solutions were measured in conventional Ubbelohde viscometers and found to be 10 ± 0.5 dl/g. These viscometers had mean shear gradients of 600–800 sec^{-1}. Extrapolation of measurements in the multibulb instrument gave intrinsic viscosities at zero shear of approximately 12 dl/g.

Electrophoresis. The change in isoelectric point of pronase-treated tropocollagen in various buffers may be read from Figure 6. Dialysis from 0.05% acetic acid solution at 4° was used to change buffers and pH, and in all cases except where calcium was present, complete precipitation above pH 4.5 occurred for the native and the sonicated tropocollagen. In calcium, the isoelectric point for the native and the sonicated tropocollagens were the same and were higher than the pronase-treated tropocollagen.

Thermal Denaturation. The melting curves in citrate buffer are shown in Figure 7. The "equilibrium" melting curve method suggested by Harrington and von Hippel (1961) was not used because it requires long periods of exposure at temperatures where cross links are known to be labile in order for "equilibrium" to be reached. Since one object in obtaining the melting curves was to determine whether the absence of cross links (in the pronase-treated tropocollagen) would change the melting point, a modification was adopted wherein complete melting curves could be obtained in several hours, and yet the values obtained should approach equilibrium values.

The scatter of points in repeated runs was quite small (*e.g.*, see 36.1° values for pronase-treated tropocollagen), and the smaller values for optical rotation of the sonicated solutions at ambient temperatures below the transition temperature was reproducible. Neither the absence of cross links nor the disruption of polymers changed the essential features of the melting curve. In particular, the threshold temperature where the last traces of structure melt out are the same in each sample tested.

The effects of sonic irradiation of tropocollagen have been studied previously (Nishihara and Doty, 1958; Hodge and Schmitt, 1958). The latter authors found that very short sonication times were sufficient to change the interaction and aggregation properties of the tropocollagen, and, in particular, to prevent the aggregation of the molecule to a 640-A native-type fiber on dialysis against 1% NaCl at 4°. This observation was confirmed with our sonicated preparations. It was

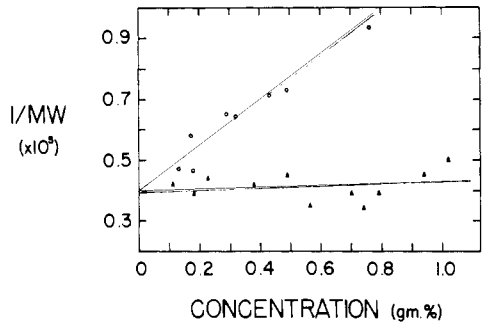

FIGURE 5: Extrapolation to zero concentration of the reciprocal of the molecular weights measured by sedimentation equilibrium. O, Pronase-treated tropocollagen, $y = 0.40\ (\pm 0.07) + 0.73x\ (\times 10^{-5})$; Δ, sonicated tropocollagen, $y = 0.39\ (\pm 0.05) + 0.035x\ (\times 10^{-5})$.

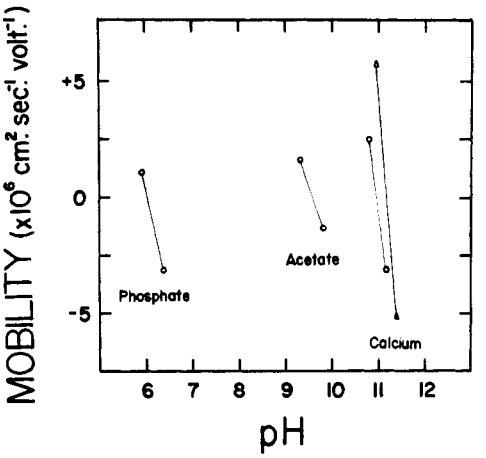

FIGURE 6: Plot of electrophoretic mobility against pH to define the isoelectric point of tropocollagen in various buffers (see Table II). O, Pronase-treated tropocollagen; Δ, sonicated and untreated tropocollagen.

found possible, however, to produce 640-A period fibers by warming solutions of the monomers in 3% NaCl to 30°. Most of such fibers had a symmetrical band structure in marked contrast to a native-type fiber (Figure 8).

The pronase-treated tropocollagen did not form fibers readily, and the fibers which were formed at relatively high concentrations of sodium or potassium chloride were characteristically either nonstriated or with only *ca.* a 90-A spacing when stained with phosphotungstic acid. Fibers with a 640-A spacing could be produced, however, by the addition of adenosine triphosphate (ATP) to low ionic strength acetate solutions at pH 4 or higher. Some physical properties for monomeric calfskin tropocollagen are summarized in Table II.

Discussion

A number of previous investigations have been made on the physical properties of tropocollagen. In most cases the investigators have gone to considerable pains to free the solutions of aggregated material by filtration or ultracentrifugation. In the experiments described in this paper the aggregates have been disrupted (rather than removed) by treating the solution with the enzyme pronase or by sonic irradiation. The molecular weights for tropocollagen determined in these experiments range from 250,000 from sedimentation equilibrium measurements, to 270,000 from the sedimentation–diffusion measurements. No correction for the charged state of the tropocollagen at pH 3.7 has been introduced in view of the recent findings of Erlander and Senti (1964) disputing the application of the conventional formula.

No difference in sedimentation equilibrium molecular weights was detected between pronase-treated and sonicated tropocollagen, although the former has been stripped of approximately 5% of its mass by the action of the pronase (Drake *et al.*, 1966). The latter factor would therefore raise the intact monomer weight to 262,000. As a mean of these figures our findings would therefore indicate 260,000 to be the molecular weight of a tropocollagen molecule. This molecular weight is significantly lower than those previously reported; the most popular current figures appear to center around 300,000 for the molecular weight of tropocollagen (*e.g.*, Rice *et al.*, 1964; Lewis and Piez, 1964). This disparity is due in no small measure to the significantly lower value for the partial specific volume measured in our experiments. Most investigators have determined the partial specific volume of denatured collagen (gelatin) and have assumed the same value holds for native tropocollagen. Our studies would refute this identity; such a change in $\bar{v}$ would be reasonable in view of the highly ordered structure of native tropocollagen, which could be expected to result in a closer packing of the amino acid residues and consequently a higher density of the protein than in gelatin. Moreover the equilibrium centrifugation experiments in CsCl reported by Fessler and Hodge (1962) demonstrate a change of density on thermal denaturation, although it must be admitted that such measurements could be misleading if changes in solvation also occur.

Our results could be in error if one or the other of the organic liquids, kerosene or bromobenzene, used to make the gradient were selectively adsorbed by the protein. However, our value of $\bar{v}$ receives some confirmation from the recently published values of Rice *et al.* (1964) who obtained a slightly higher value than ours in a gradient using different organic liquids. The values quoted by those authors, however, should be revised downward to 0.676 in order to be compared with our value, since their concentrations were based upon a nitrogen content of 17.0%.

Our value for $\bar{v}$ and hence for the molecular weight of tropocollagen depends upon our calculated nitrogen content. The range of values in the literature (17.4–

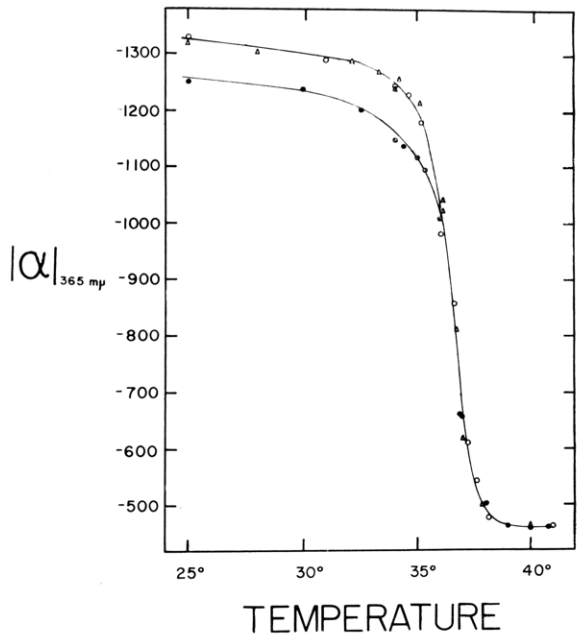

FIGURE 7: Melting curves for native (○), sonicated (●), and pronase-treated (△) tropocollagen solutions (0.1–0.2%) determined by measurement of the specific optical rotation at the 30-min interval in each temperature step.

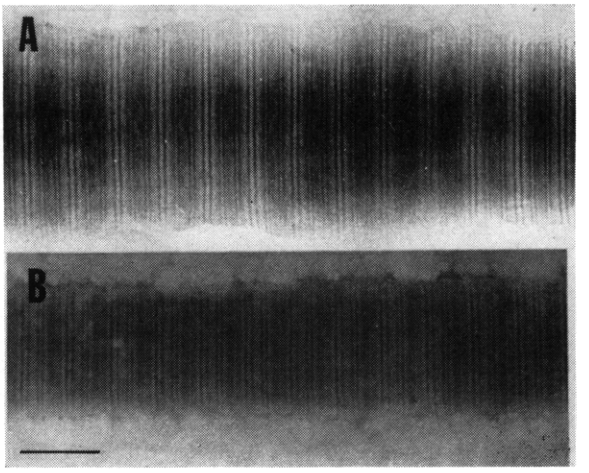

FIGURE 8: Electron micrographs of fibrous aggregates of tropocollagen before and after sonic irradiation. A. A: symmetrically banded 640-A fiber formed by warming sonicated tropocollagen (0.1%) in 3% sodium chloride solution to 30°. B: A native-type fiber formed in the cold (4°) from calfskin tropocollagen when dialyzed against 1% sodium chloride solutions. Both fibers are positively stained using 0.1% phosphotungstic acid at pH 4. The bar on the plate represents 1000 A.

18.6% for mammalian collagens, see Eastoe and Leach, 1958) attests the difficulty of precisely determining the nitrogen content of purified, dried, ash-free collagen and we have preferred to calculate this figure from the amino acid composition. If this assumed value is too low, then $\bar{v}$ is lower than 0.655 and the calculated molecular weight will also be lowered.

Some of the difference between our molecular weights and those of others must also be attributed to the presence in most tropocollagen solutions of polymeric aggregates of molecules. The presence of linear polymers is attested by the viscosity of the normal, untreated tropocollagen solutions in which values are usually higher than the figure 10 dl/g attributed by us and by Veis and Drake (1963) (9.7 dl/g) to solutions of monomers. The susceptibility of these aggregates to sonic irradiation also provides evidence of linear polymerization (albeit with some overlap, Hodge and Petruska, 1963) since the disrupting action of sonic irradiation is by hydrodynamic shearing forces (Gooberman, 1960; Freifelder and Davison, 1963) which would not be expected to separate lateral aggregates of small dimension.

It is perhaps worthy of note that linear polymers would be difficult to remove from a tropocollagen solution by centrifugation. The presence of such polymers will necessitate a revised interpretation of physical measurements by light scattering and other colligative methods on tropocollagen solutions.

The value for the molecular weight of tropocollagen which we propose is compatible with the X-ray diffraction measurements of Cowan *et al.* (1955) and Ramachandran (1963), although these data are still under review (see, Davies, 1965). If tropocollagen consists of a continuous triple helix with a repeat distance per residue (in stretched fibers) of 3.10 A while the total length of the monomer is (Hodge and Petruska, 1963) 4.4 times the low angle periodicity, *i.e.*, 700 A for a wet stretched fiber, then each molecule contains approximately 3000 amino acid residues. The average residue weight from our own analyses (Rubin *et al.*, 1965) is 91.2. These figures combine to give a molecular weight of 270,000 for native tropocollagen, *i.e.*, molecules after secretion from the synthesizing cells and which have been provided with a complement of telopeptides which makes possible axial and lateral aggregation in normal fibrogenesis.

The electrophoresis experiments were performed to obtain data on the effect of various buffers commonly used with collagen, since native tropocollagen is insoluble in most buffers above pH 4.5 while the pronase-treated tropocollagen is very soluble in buffers throughout the pH range. Comparison of the two materials directly was possible only in the calcium solutions where the calcium presumably binds primarily with the carboxyl groups. The higher isoelectric point of the native tropocollagen reflects its higher content of arginine residues, but the possibility that it has sites which preferentially bind the guanidine used to maintain the pH must also be recognized.

The extensive binding of ions in general is shown by a marked change in isoelectric point depending upon the

TABLE II: Physical Characteristics of Monomeric Calfskin Tropocollagen Solutions.

	Sonicated	Pronase-Treated
Intrinsic viscosity, $[\eta]$ (100 ml/g)[a]	10	10
Partial specific volume, $\bar{v}$ (ml/g)[a]	0.65_4	0.65_5
Diffusion coefficient $D_{4°}$, 0.15 M citrate (cm^2/sec $\times 10^7$)		0.43_6
Sedimentation coefficient $s^0_{20,w}$ (S)	3.4[a,b]	3.4[a,b] 3.8[a,c] 3.3[a,d]
Mol wt, M		
Sedimentation–diffusion[a]		268,000
Sedimentation equilibrium[a]	260,000	262,000[e]
Calculation (see text)	270,000	
Isoelectric point, pH		
Phosphate[f]	Ppt	6.0
Acetate[g]	Ppt	9.6
Calcium[h]	11.3	11.0
Denaturation temp (°C)[a]	36.4	36.4

[a] 0.15 M citrate, pH 3.7. [b] Sedimentation at 20°. [c] Sedimentation at 4°. [d] Sedimentation of denatured α components at 40°. [e] 250,000 mol wt plus 5% split by pronase digestion. [f] 0.08 M monobasic potassium phosphate–potassium hydroxide. [g] 0.1 M sodium acetate–0.001 M Tris (or glycine or veronal). [h] 0.03 M calcium acetate–0.001 M guanidine.

specific ion environment. A marked effect of ions on the isoionic point (pI) of collagen has been reported (Veis *et al.*, 1958), and a similar but smaller effect of ion binding for Na^+, Cl^-, Ba^{2+}, and SO_4^{2-} has been shown by Bensusan and Hoyt (1958) whose soluble calfskin tropocollagen had an isoelectric point of 8.3 in a 0.1 M Tris buffer.

Three groups of investigators have recently published observations on *in vitro* fiber formation. Candlish and Tristram (1964) found that amino acids exerted a stabilizing effect on the formed fibers, and Kühn *et al.* (1964) used a nonprotein fraction from early extracts of calfskin collagen to enhance fibrogenesis. Our data emphasize the potential importance of ion binding *in vivo* for both collagen transport and for fibrogenesis, but the data also indicate that great caution must be used in interpreting results since a great many charged molecules may be operative in *in vitro* experiments, but have little relationship *in vivo*.

In the third study, Hafter and Hörmann (1963) and Hafter (1964) noted an inflection point at pH 4.2–4.5 in heat-precipitating fibers and suggested that this coincides with the isoelectric point. Since our studies and those of others indicate a higher isoelectric point for native tropocollagen, it is more likely that the pH 4.2–4.5 range reflects a titration point of one or more specifically located carboxyl groups which enhance fibrogenesis. We have noticed that aggregates produced by ATP change from the typical segment-long-spacing form to 640-A spacing fibers in a qualitatively similar pH range (pH 3.8–4.3).

In regard to the melting curves, Flory (1956) predicted that the imposition of cross linkages on the ordered tropocollagen molecule leads to an increase in the denaturation temperature, and such an increase was confirmed by Veis and Drake (1963) after the introduction of synthetic cross links with formaldehyde into native Buffalo fish tropocollagen. The expected decrease in the denaturation temperature for the pronase-treated tropocollagen in which all of the cross links have been removed was not observed, however, and this probably indicates that the average number of cross links in soluble calfskin tropocollagen is quite low, perhaps less than 2 cross links/molecule. Such a value for soluble collagen would agree with the value of 1.3 cross links/α chain (4/molecule) calculated for insoluble tendon collagen (Cater, 1963). Perhaps it is worth noting that the minimum number of cross links necessary to make an infinite network is only 1 cross link/α chain (3/molecule).

Area measurements on the schlieren diagrams of tropocollagen before and after sonic irradiation (Figure 8, Drake *et al.*, 1966) show that the α component is enriched by 15% of the protein at the expense of the other components. If dimers are held together by α–α bridges, then the appearance of 15% more α implies that 45% of the original tropocollagen solution is in a dimeric form. If very long strands are integrally linked by α–α bonds, then 30% of the original tropocollagen solution is in a polymeric state. If dimers are linked by more than one α–α bridge between respective α chains, then the percentage of dimers could be as low as 15%. These calculations, therefore, suggest that between 15 and 45% of the tropocollagen solution is in a polymeric form.

The intrinsic viscosity of the monomers is 10 dl/g. A dimer linked with a 10% overlap (Hodge and Petruska, 1963) would have a length 1.9 times the monomeric length and an intrinsic viscosity of approximately 30 dl/g (Mehl *et al.*, 1940). The observed viscosity of native tropocollagen solutions ranges from 16–20 dl/g in our hands, and this figure would be compatible with a content of approximately 45% dimers. Moreover, since the viscosity increment of long molecules increases as a high power of the length of these molecules, it is apparent from these crude calculations that few very long polymers can be present in the tropocollagen solutions.

The initial small decrease in specific optical rotation values for tropocollagen solutions under sonic irradiation is similar to that observed in the early stages of pronase digestion (Drake *et al.*, 1966). That decrease was ascribed to the presence of the split telopeptides which no longer contributed to the "helix content,"

for during subsequent dialysis the specific optical rotation returned almost to its original value. We would suggest that a second effect of sonication, therefore, is a "denaturing" of helical telopeptides which re-form hydrogen bonds in a random manner after sonication. This interpretation receives support from the fact that the decrease in specific optical rotation occurs after short sonication times (*i.e.*, 0.5 hr) and does not change appreciably at long sonication times (*i.e.*, 6 or more hr). If a general denaturation of the tropocollagen was occurring, one would expect a continuing fall in both specific optical rotation and in intrinsic viscosity with time of sonication.

Such an interpretation would be in accord with observations that very short sonication times are sufficient to reduce the ability of the tropocollagen to interact to form 640-A native-type fibers in 1% NaCl solutions (Hodge and Schmitt, 1958). Bensusan (1960) has provided evidence that the rate of fiber formation is proportional to the activity of ionizable groups on the tropocollagen molecule. Thus, the formation of a specific configuration (*i.e.*, the native fiber) by untreated soluble calfskin tropocollagen molecules must mean that at least one sharply defined maximum of charge attraction between molecules exists when they are correctly oriented with respect to each other. Such a maximum would account for the tendency of such molecules to orient in solution (*i.e.*, the thixotropic properties of collagen solutions), and for the property of untreated tropocollagen to precipitate as native-type fibrils under a range of solvent and temperature conditions. The specific configuration required to produce such a maximum is postulated to involve at least some of the telopeptides whose charged groups are arranged along the length of the molecule. A relative shift in position of some of these charged groups (*i.e.*, a new position assumed by the telopeptide as it re-forms hydrogen bonds) might be expected to impair fiber formation along with reducing the specific optical rotation. Moreover, we would predict that any operation involving the telopeptides (such as the thermal denaturation–renaturation of γ-tropocollagen) would result in a lower specific optical rotation for the "native" renatured solution than was measured for the starting material before denaturation, and, in fact, this already has been observed (Altgelt *et al.*, 1961; Drake and Veis, 1964).

The formation of a symmetrically banded 640-A fiber from sonicated solutions, of unstriated and 90-A spacing fibers from pronase-treated tropocollagen, and of 640-A spacing fibers from pronase-treated material by the use of ATP was observed early in these studies but a rationale to account for them was not apparent at that time. The production of normal segment-long-spacing aggregates requires that the molecules be in register and specifically oriented (Hodge and Schmitt, 1960), and the formation of native fibers (as discussed above) requires a specific "head-to-tail" orientation and a specific lateral displacement. Molecules in register but randomly oriented will produce symmetrical segment-long-spacing aggregates (Drake *et al.*, 1966), and the formation of symmetrically banded 640-A fibers (by analogy) arises from a random "head-to-tail" orientation with the specific lateral displacement being maintained. The unstriated or small-spacing fiber, then, is formed when both the orientation and the lateral displacement are less regular. In the case of the production from pronase-treated tropocollagen of 640-A fibers with ATP, the requisite lateral displacement is produced because the phosphates bind preponderantly with the guanidine groups of arginine (Kühn *et al.*, 1958), and Bensusan *et al.* (1962) have shown that the arginine groups occur in five rather discrete "bands" along the length of the molecule.

Kühn *et al.* (1964) have reported the formation of both symmetrical and small striation fibers from enzyme-treated and from "highly purified" tropocollagen preparations. Since their "highly purified" tropocollagen has a very low tyrosine content, we would surmise that both of the preparations they used had been subject to protease action; the fiber formation they report, therefore, probably arises because of the removal of particular charge groups by enzyme scission of telopeptides.

In summary, it has been shown that most soluble tropocollagens contain linear polymers; that either limited digestion by pronase or sonication at low energies may be used to produce monodisperse solutions; and that physical measurements on such solutions provide new values for some of the physical parameters of tropocollagen. In addition, electrophoresis data have been presented which show that tropocollagen will readily bind both anions and cations with a large resultant shift in its isoelectric point, and a postulate has been made concerning a role of telopeptides in the orientation of molecules for precipitation as aggregates or fibers.

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