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Some Properties of the Products of Reaction of Tadpole Collagenase with Collagen*

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ABSTRACT: Cleavage products of the action of tadpole collagenase on mammalian and tadpole collagens have been isolated and separated by ammonium sulfate fractionation, and have been analyzed for amino acid composition, size, shape, molecular weight, conformation, and stability. Molecular weight determinations by sedimentation equilibrium ultracentrifugation agree closely with the earlier observations that the two fragments produced by enzyme action represent three-quarters and one-quarter of the collagen molecule. Values for the number-average molecular weight for the small fragment (TC^B), the larger fragment (TC^A), and the intact molecule (TC) were 70,000, 202,000, and 298,000, respectively. Calculations for molecular length and rigidity from viscosity measurements were consistent with a rigid rod structure for two fragments, one, one-quarter, and the other, three-, quarters of the intact molecule. Optical rotatory dispersion measurements indicated that helical content was preserved in the fragments. The fragments heat denatured more readily than collagen; at acid pH T_m values were 32 and 29° for TC^A and TC^B, respectively, as compared with 36° for TC (calf skin). Tadpole collagen at acid pH gave T_m values of 23.2, 24.5, and 29°, respectively, for TC^B, TC^A, and TC; at neutral pH the values were 1–2° higher. TC^B and TC^A differed significantly from each other in the content of 15 of the amino acids; only glycine, glutamic acid, and aspartic acid showed less than 10% difference. The total imino acid percentage was higher in TC^B than in either TC^A or TC, yet the denaturation temperature was lower. Other points characteristic of the fragments as compared to native collagen were increased solubility at neutral pH and greater susceptibility to degradation by trypsin. A hypothesis on the mechanism of physiologic resorption of collagen based on these findings is proposed.

An enzyme isolated from the medium of cultures of living tadpole tissues cleaves the native collagen molecule into two fragments in a highly specific manner at physiologic pH and at a temperature below that of substrate denaturation (Gross and Lapiere, 1962; Lapiere and Gross, 1963; Nagai *et al.*, 1966; Gross and Nagai, 1965; Kang *et al.*, 1966). The enzyme has been purified and partially characterized (Nagai *et al.*, 1966). Electron microscope studies of segment long spacings (SLS)[1] indicated that the molecule is severed one-quarter of the distance from the "B" end (Gross and Nagai, 1965). There is no further attack on the two intact fragments, and preliminary analyses of the terminal amino acids indicate that no more than three peptide bonds per collagen molecule are broken (Nagai *et al.*, 1964), probably one in each polypeptide chain. Kang *et al.* (1966) have reported the amino acid analysis and molecular weights of the separated polypeptide chains obtained by denaturation of the reaction products and isolation by chromatography. In this paper we describe the separation of the undenatured reaction products into the large (TC^A) and small (TC^B) fragments, and measurements of their size, shape, molecular weight, amino acid composition, and conformational stability.

Experimental Procedures

The tadpole enzyme was prepared and purified as described by Nagai *et al.* (1966) with the exception that the starch block electrophoresis and DEAE-cellulose chromatography steps were omitted, since it was found that the characteristic enzyme action was unchanged by further purification and that the more

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[1] Abbreviations used: SLS, segment long spacing; TC^A and TC^B, large and small fragments, respectively, of TC (the intact molecule); TCA, trichloroacetic acid; ATP, adenosine triphosphate; PTA, phosphotungstic acid.

highly purified enzyme was unstable.

Preparation of Collagen Substrate. Neutral and acid-extractable collagens were obtained from calf skin and guinea pig skins and from tadpole tail fin and back skin. The cleaned and minced tissues were initially extracted three times in cold 1 M NaCl to obtain the neutral extracted fraction. The residue was then re-extracted with 20 volumes of 0.5 M acetic acid. ^{14}C-Labeled collagen was prepared from guinea pig skin as described elsewhere (Lapiere and Gross, 1963). The extracts were clarified by centrifugation and filtration and the collagen was purified by a TCA–ethanol procedure (Gross, 1958); in some instances it was reprecipitated by dialysis against 0.01 M disodium phosphate. The resultant collagen was redissolved in 0.1 M acetic acid, dialyzed, lyophilized, and stored dry at −20°. For use, it was dissolved in phosphate buffer, $\Gamma/2 = 0.4$, pH 7.6, by shaking overnight on a wrist shaker at 5° to a final concentration of about 0.4%, then dialyzed against 0.4 M NaCl. The highly viscous solutions were diluted with equal volumes of 0.05 M Tris buffer, pH 7.5, containing 0.001 M $CaCl_2$, then clarified by high-speed centrifugation.

Preparation of Reaction Mixture. Typical reaction mixtures contained 0.2% collagen, 0.002% enzyme, 0.2 M NaCl, 0.025 M Tris-HCl buffer (pH 7.5), and 0.0035 M $CaCl_2$. The mixture was incubated at 20° for 24 hr and the rate of reaction followed in an Ostwald viscometer having a flow time of 30 sec for water at 20°. After the viscosity had become constant, within 24 hr, at about 30–50% of the starting value, EDTA (sodium), pH 7.5, was added to a final concentration of 0.005 M to stop the reaction. (This addition was later found to be unnecessary.)

Separation of TC^A and TC^B. To the reaction mixture in an ice bath solid ammonium sulfate was added slowly with constant stirring to 12% saturation (6.75 g/100 ml). Saturation of distilled water at ice-bath temperature with ammonium sulfate was established at 62.6 g/100 ml. After 1 hr the precipitate was collected by centrifugation and the ammonium sulfate concentration in the supernatant was increased to 15%. Precipitates were collected at 12, 15, 20, 30, and 50% ammonium sulfate saturation. After washing at the same $(NH_4)_2SO_4$ concentration and recentrifugation, each precipitate was dissolved in 0.1 M acetic acid, dialyzed against 0.005 M acetic acid, and lyophilized.

Disc electrophoresis and electron microscopy of SLS formed from the reaction products were used to identify the components and to monitor purity of the fractions. The recovery of collagen in the precipitate was measured by either hydroxyproline content or by radioactivity when ^{14}C-labeled neutral extracted collagen was used. The redissolved precipitates were examined by disc electrophoresis after denaturation by heating to 40°.

Disc Electrophoresis. The disc electrophoretic methods for collagen are described by Nagai *et al.* (1964). The earlier recipe was modified in order to obtain better resolution of the various collagen components as described below. Kulonen *et al.* (1965) have pointed out the superior resolving power of collagen components on starch gel at pH 5.6 than at lower pH. Polyacrylamide gels prepared at 7.5 or 10% concentration were used in these studies. A current of 2–3 ma was applied to the gels for 3 hr at room temperature. Under these conditions the $\alpha 1$, $\alpha 2$, β_{11}, β_{12}, and γ components were completely separated.

Stock Solutions (keep cold). In the *upper gel* the mixture a:b:c:H_2O = 1:2:1:4: (a) KOH (1 N, 6.8 ml), acetic acid (1 N, 8.7 ml), and temed (0.3 ml) (*N,N,N′,N′*-tetramethylethylenediamine) (add H_2O to 100 ml, pH 6.2); (b) acrylamide (10.0 g) and bis (*N,N′*-methylenebisacrylamide) (2.5 g) (add H_2O to 100 ml); (c) riboflavin (4 mg) (add H_2O to 100 ml).

In the *lower gel* the mixture d:e:f:H_2O = 1:2:4:1: (d) KOH (1 N, 13.6 ml), acetic acid (1 N, 26.5 ml), and temed (0.5 ml) (add H_2O to 100 ml, pH 5.3); (e) acrylamide (30.0 g) and bis (0.8 g) (add H_2O to 100 ml); (f) $(NH_4)_2S_2O_8$ (0.15 g) (add H_2O to 1 l.).

In the *upper tray* the mixture g = 150 ml, h = 50 ml, and H_2O (to 2.1): (g) β-alanine (35.6 g) and acetic acid (1 N, 3.0 ml) (add H_2O to 1 l., pH 5.5); (h) Tris (4.85 g) and acetic acid (glacial) (2.4 ml) (add H_2O to 1 l., pH 5.5).

In the *lower tray* the mixture i = 50 ml and H_2O (to 1 l.): (i) potassium acetate (33.3 g) and acetic acid (glacial) (5.1 ml) (add H_2O to 1 l., pH 5.3). Use 2 l. of tray buffers.

Viscosity Measurements. Measurements of reduced viscosity as a function of concentration of collagen were made at 20° in a Cannon Ubbelohde semimicro dilution viscometer (flow time of 90 sec for water at 20°). Concentrations of native collagen, TC^A, and TC^B ranged from 0.01 to 0.1% in 0.05 M acetate buffer, pH 4.8, containing 0.1 M sodium chloride. The initial concentrations were measured by a microhydroxyproline determination (Bergman and Loxley, 1963) and the collagen content was calculated on the basis of 13% hydroxyproline.

Sedimentation Equilibrium Measurements. A Spinco Model E analytical ultracentrifuge with temperature maintained at 5°, equipped with a Rayleigh interference optical system and aligned by the method of Richards and Schachman (1959), was employed.

The high-speed equilibrium technique of Yphantis (1964) was used for estimating the molecular weight. Measurements of fringe displacement as a function of the distance, x, from the axis of rotation were made with a Nikon Model 6 optical microcomparator. Two initial concentrations were used: 0.01 and 0.02% collagen in 0.05 M acetate buffer, pH 4.8, containing 0.1 M NaCl. Concentration was estimated at 0.1-mm intervals using an average of seven fringes. Speeds of 12,590 for TC, 14,290 for TC^A, and 25,980 for TC^B were employed and the samples were run in 12-mm cells for 15–20 hr until the concentration at the meniscus approached zero. Weight-average (M_w) and number-average (M_n) molecular weights were determined from the plot of log C_x *vs.* x^2; number-average molecular weights were determined by using the equations of Yphantis (1964). A partial specific volume of 0.66

for native collagen, based upon recent measurements of Davison and Drake (1966), was used in the molecular weight calculations.

Optical Rotation and Denaturation. A Cary 60 automatic spectropolarimeter was used for measurements of optical rotatory dispersion in the wavelength range between 260 and 187 mμ at concentrations of TC, TC^A, and TC^B ranging from 0.01 to 0.1% in 0.01 M HCl at pH 2.0. Temperature was maintained at 10–15°.

The denaturation characteristics of the collagen fragments and native collagen were compared by measuring optical rotation in 1-dm water-jacketed cells at 364, 404, and 464 mμ using a Rudolph Model 200 photoelectric spectropolarimeter equipped with an oscillating polarizer. The collagens were dissolved at 0.1–0.2% concentration in several different buffers, dilute HCl (pH 3.5), 0.25 M sodium citrate (pH 3.7), 0.15 M sodium acetate (pH 3.5), and amphibian Tyrode solution (Nagai *et al.*, 1966) (pH 7.5). Native collagen at neutral pH could be measured only in 0.4 M phosphate, pH 7.6, containing 0.2 M arginine to prevent fibril formation on warming (Gross and Kirk, 1958). The temperature was raised 1° at 20-min intervals. Renaturation of denatured TC, TC^A, and TC^B was accomplished by abruptly lowering the temperature of the denatured protein to 25° after complete gelatinization at 45°; the increase in negative optical rotation was monitored for 40 hr.

Action of Trypsin on Isolated Fragments. The susceptibility of isolated TC^A, TC^B, and TC to tryptic digestion was examined using 0.1% solutions of calf skin and tadpole collagens in 0.15 M Tris–NaCl, pH 7.6, and 0.01% crystallized trypsin was added at 5°. These preparations were examined in a Rudolph spectropolarimeter at 365 mμ, the temperature being raised 5° at intervals of 30 min, and the optical rotation was recorded every 5 min during the 30-min intervals at constant temperature.

Preparation of Segment Long Spacing for Electron Microscopy. Samples of TC, TC^A, and TC^B were dissolved in 0.05 M acetic acid in concentrations between 0.01 and 0.2%. After clarification by centrifugation and dialysis against the same solvent for a period of 24–48 hr, the SLS structures were formed by adding a 1% aqueous solution of ATP (free acid) to a final concentration of 0.4%. Drops of the cloudy suspension were placed on collodion-coated grids; the grids were drained and positively stained with 1% PTA. All stages of specimen preparation must be done in the cold. An RCA EMU 3G electron microscope was used for taking pictures, usually at 26,000× or 50,000× magnification.

Results and Discussion

The composition and amount of the reaction products isolated at various concentrations of ammonium sulfate are described in Table I. At low $(NH_4)_2SO_4$ concentra-

TABLE I: Recovery of Collagen Fragments from Reaction Mixture of Collagen and Tadpole Collagenase by Ammonium Sulfate Fractionation.

% $(NH_4)_2SO_4$ Satn	Ppt (mg)[a]	% Recov	Compn by Disc Electrophoresis	Total (cpm)	% Recov
		Calf Skin			
0–12	33	18.3	TC + TC^A		
12–15	64	35.6	TC^A		
15–19	12.5	7.0	TC^A + TC^B		
19–30	21	11.7	TC^B		
30–50	0.5	0.3	(Enzyme)		
50	Trace	–	–		
Total	131	73	–		
		[^{3}H]Proline-Labeled Guinea Pig Skin			
0–20	14.34	79	TC^A (TC, TC^B)	3740	75
20–30	1.91	11	TC^B	640	13
30	0.23	1.3	–	190	4
Dialysate	0.096	0.5	–	120	2.4
Total	16.6	92	–	4690	94
		Guinea Pig Skin			
0–20	11.51	70.5			
20–30	1.56	9.5			
30	0.08	0.5			
Dialysate	0.07	0.4			
Total	13.22	81			

[a] Precipitate was dialyzed and lyophilized.

tions (0–12%) about 18% of the total collagen was recovered as a mixture of native molecules and a small amount of TC^A. Between 12 and 15% saturation the bulk of TC^A precipitated almost quantitatively and was quite pure. Between 19 and 30% saturation, TC^B was recovered in essentially pure form; above 30% only traces of material were obtained. In this particular experiment using calf skin collagen there was a loss of about 30% of the material. In other experiments performed on salt-extracted guinea pig skin collagen the recoveries were better, 92% in one and 81% in the other. In these cases the 0–20% precipitate contained TC^A almost exclusively. Where the reaction was incomplete residual intact collagen was precipitated in the 0–12% saturation fraction. The bulk of TC^B, essentially pure, was obtained between 20 and 30% saturation. Some losses were sustained during washing of the precipitate and in lyophilization. Less than 0.5% of the collagen in the reaction mixture was dialyzable as measured by hydroxyproline, although about 2% of the radioactivity of [^{14}C]collagen was obtained in the dialysate.

Disc electrophoresis of the polypeptide chains obtained by denaturation of TC^A and TC^B confirmed the purity of the preparations. Figure 1 compares the patterns obtained from isolated TC^A and TC^B with that of control collagen containing inactivated enzyme and also that of an unfractionated reaction mixture (see also Gross and Nagai, 1965). The nomenclature of Kang *et al.* (1966) for designating the subunit fragments is used here. The control pattern shows the usual $\alpha 1$ and $\alpha 2$ bands corresponding to single polypeptide chains of 100,000 molecular weight and the β_{11} and β_{12} bands corresponding to the cross-linked double chains. The $\alpha 1$ band appears to be partially resolved into two components.

Splitting of the $\alpha 2^A$ band was occasionally observed, as shown in Figure 1. In the patterns of the short pieces (TC^B), $\alpha 1^B$ and $\alpha 2^B$ are widely separated from each other and from the TC^A components. Each is clearly a double band with the faster component more heavily stained and broader in both cases. There is more $\alpha 1^B$ than $\alpha 2^B$ which is also expected since there are two chains of $\alpha 1$ for each of $\alpha 2$. A third faster band appeared occasionally in the $\alpha 2^B$ region.

It is clear from the electrophoretic patterns of the reaction products that the enzyme does not break the cross-links of the β and γ components. The occasional splitting of each of the new bands could be explained if both $\alpha 1$ and $\alpha 2$ chains each represent two slightly different species. The $\alpha 1$ fraction of codfish skin collagen is a mixture of two components differing in amino acid composition and designated $\alpha 1$ and $\alpha 3$ by Piez (1965). An even more subtle heterogeneity of the $\alpha 1$ fraction of rat skin has been noted by Piez *et al.* (1966) in the oxidation of the ϵ-NH_2 group of one lysine residue at the N-terminal end of a portion of the $\alpha 1$ chains. Alternatively, the fragments may be heterogeneous because of continued slow enzymatic action after the initial cleavage. There is some indication of this in the work of Kang *et al.* (1966) on the isolated, denatured chain fragments.

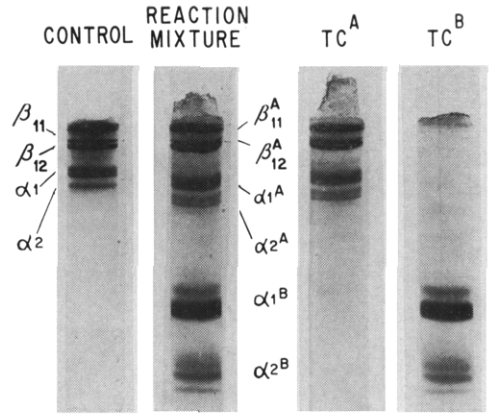

FIGURE 1: Disc electrophoretic patterns of thermally denatured calf skin collagen reaction products of tadpole collagenase digestion compared with control collagen.

Electron Microscopy of Segment Long Spacing of TC^A and TC^B. Purified TC^A formed SLS structures readily; no contamination from full-length or quarter-length segments of the TC^B segments was observed. Both forms of SLS were identical with those formed from the whole reaction mixture, shown previously by Gross and Nagai (1965). In some preparations the TC^A segments formed dimers with the cut ends in apposition. In other samples there were occasional segments with "A" ends in close contact. The formation of fibers by linear association of SLS occurred occasionally. In such instances there was usually alternating apposition of "A" ends and cut ends, but rarely was there continuous polarization in one direction. TC^B, on the other hand, showed a much richer variety of SLS aggregates. Single SLS was rare. The most common form was a dimer; in some preparations the cut ends were in apposition whereas in others the terminal "B" ends were abutting. In a number of instances four or more segments were aggregated linearly to give the appearance of a polarized periodic fibril. At high TC^B concentration, *ca.* 0.2%, two-dimensional sheets of TC^B (SLS) were found in which all the segments were polarized in the same direction and overlapping each other, to give rise to a period of about 300 A. These structures will be described and analyzed in detail elsewhere.

Molecular Weight and Shape. For the native tropocollagen molecule, TC, the plot of log C_x *vs.* x^2 (Figure 2) indicates some heterogeneity as shown by the upward curvature. The same effect, although less marked, is noted for TC^A, but in the case of the short pieces, TC^B, the plot exhibits downward curvature, indicative of slight nonideality. For TC^B, molecular weight is almost independent of position in the cell, indicating homogeneity of the preparation (Table II). Values of M_w and M_n extrapolated to zero concentration agree well for both TC^A and TC^B. This was not the case for TC, where M_w is higher than M_n probably as a result of

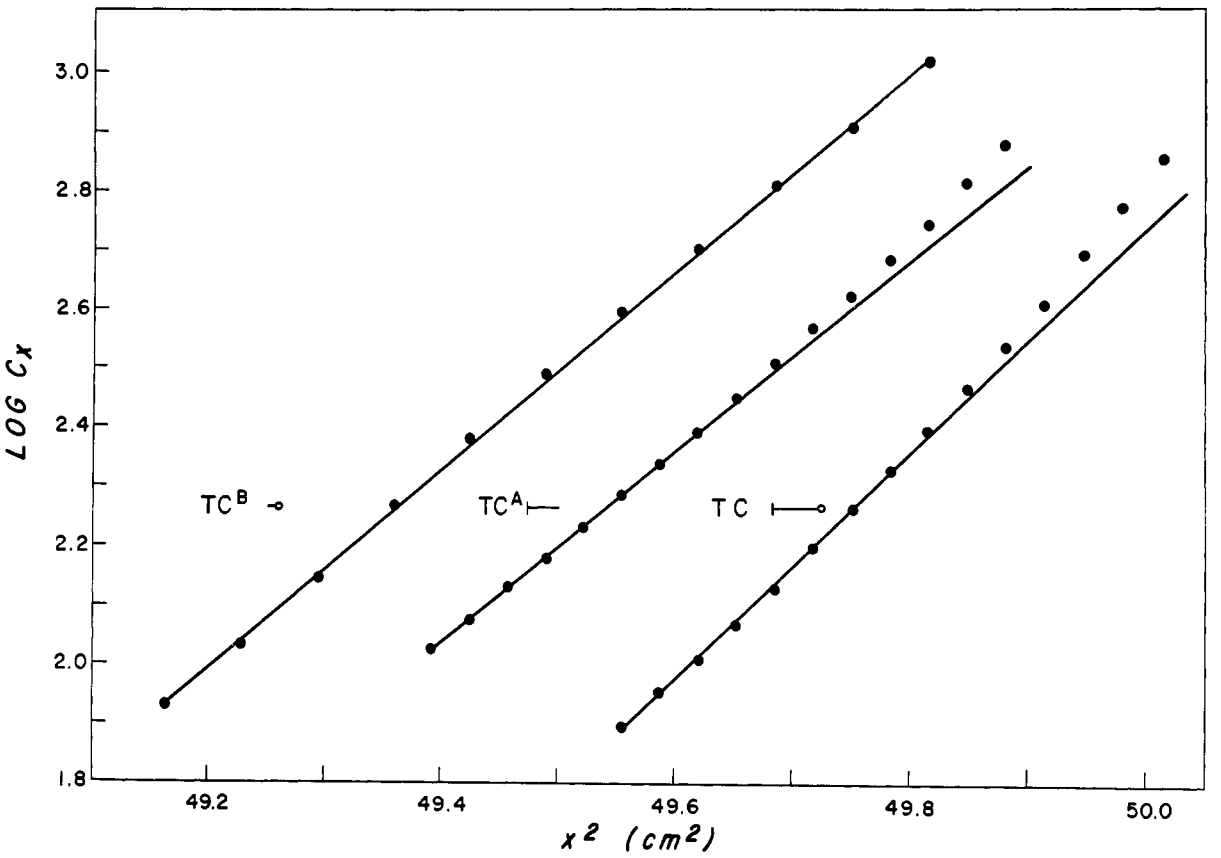

FIGURE 2: Sedimentation equilibrium analysis for the two calf skin collagen fragments, TC^B and TC^A, isolated from the reaction mixture compared with that for control collagen, TC.

TABLE II: Molecular Weights of TC, TC^A, and TC^B Calculated from $1/M$ *vs.* C Plot.

	TC		TC^A		TC^B	
x^a	M_n	M_w	M_n	M_w	M_n	M_w
$C \rightarrow 0$	302,000	319,000	209,000	210,000	75,000	76,200
	302,000		214,000	214,000	73,400	
	303,000	323,000	216,000	216,000	73,800	73,400
	307,000	334,000	216,000	222,000	76,700	74,900
	310,000	351,000	217,000	220,000	74,400	73,200
	317,000	358,000	218,000	226,000	73,700	70,000
	329,000	367,000	218,000	233,000	72,500	69,400
	328,000	368,000	220,000	232,000	71,700	68,300
	333,000	380,000	224,000	249,000	70,900	68,500
	338,000	388,000	227,000	248,000	69,800	
	346,000	401,000	230,000	252,000	70,000	
	353,000	424,000	231,000	257,000		
	366,000	445,000		267,000		
b^b	395,000	550,000	250,000	312,000	70,000	68,000
M_n^c	351,800		232,400		71,500	

[a] Column represents increasing distances along cell axis for which molecular weights were derived. [b] Molecular weight at bottom of cell. [c] Mean number-average molecular weight over the entire cell.

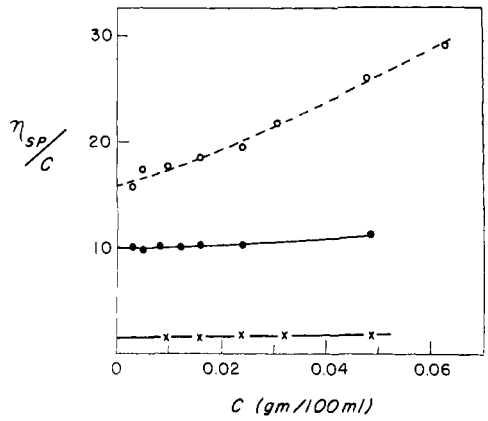

FIGURE 3: Plots of reduced viscosity *vs.* concentration for the two calf skin collagen fragments isolated from the reaction mixture and control collagen. O---O, TC; ●---●, TC^A; and ×---×, TC^B.

aggregation. A summary of the data is presented in Table II in which M_w and M_n are related to the distance from the axis of rotation. For TC^B values of mean molecular weight over the whole cell, the weight at the bottom of the cell and that obtained from the least-square plot extrapolated to zero concentration are in good agreement. From the number-average molecular weights calculated for $C \rightarrow 0$, the sum of TC^A and TC^B is somewhat lower than that obtained for the native molecule TC, but very close to the value of 286,000 calculated for tropocollagen from the molecular length, average residue translation, and the amino acid composition (Lewis and Piez, 1964). Reduced viscosities as a function of concentration for the two isolated fragments and the intact molecule are compared in Figure 3. In the case of TC^B and TC^A the curve is very flat, indicating much less molecular interaction at the same concentrations than was the case for TC.

Calculation of length from the equation of Simha (1940) (see Yang 1961) yields values of 3050 A for TC,

$$L = 2a = \frac{(600)^{1/3}}{\pi N}([\eta]M)^{1/3}(p^2/\nu)^{1/3}$$

2300 A for TC^A, and 800 A for TC^B.

If this equation is corrected to apply to a highly asymmetric rigid cylinder of the same volume and axial ratio by substituting $V_e = \pi d^2 L/4$ (cylinder) for $4/3\pi ab^2$ (ellipse) in $[\eta] = NV_e\nu/100M$, one obtains $L = ((400)^{1/3}/\pi N)([\eta]M)^{1/3}(p^2/\nu)^{1/3}$, where $p = a/b = L/d$.

This equation gives values of 2610, 1970, and 680 A for TC, TC^A, and TC^B, respectively, which are

[2] L = molecular length; p = axial ratio; $a/b = L/d$ = long axis/diameter of oblate ellipsoid or cylinder; M = molecular weight; N = Avogadro's number; V_e = molecular volume (dry); and ν = viscosity increment.

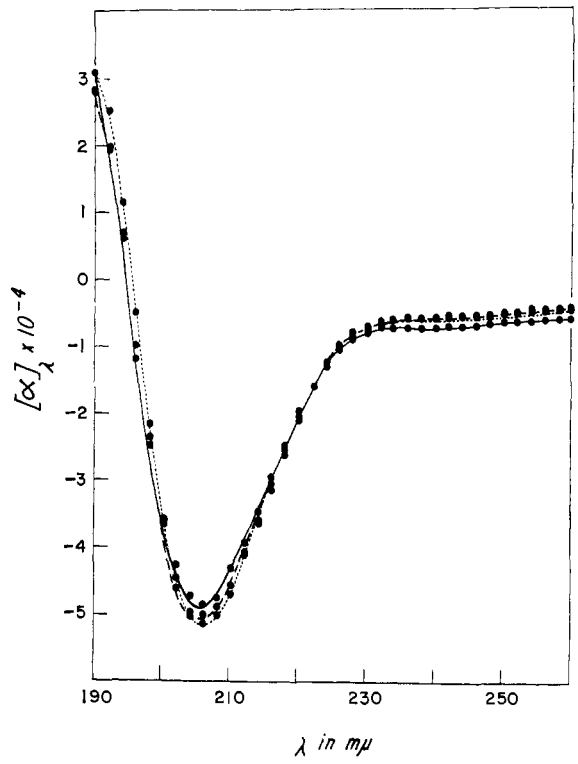

FIGURE 4: Optical rotatory dispersion curves for isolated collagen fragments and control collagen. ●——●, TC; ●---●, TC^A; and ●···●, TC^B.

closer to the lengths derived from measurements of SLS in the electron microscope (Gross and Nagai, 1965). As pointed out by Boedtker and Doty (1956), for a highly asymmetric cylinder such as collagen, water of hydration does not contribute significantly in the calculations of length.

A measure of the rigidity and rodlike character of a macromolecule in solution may be obtained from viscosity measurements by employing the equation of Houwink (1940), $[\eta] = KM^\alpha$, where K and α are constants for a given solute and solvent system and α is an index of flexibility. Values of α obtained for flexible chains, stiff polymers, and rigid ellipsoids or cylinders are, respectively, 0.5–0.8, 0.8–1.2, and 1.7–2.0 (see Yang, 1961, for discussion). If molecular weights of TC, TC^A, and TC^B are represented as $4M$, $3M$, and $1M$, α may be calculated from any two since the intrinsic viscosities are known. Thus, $\alpha = 1.67$ from the simultaneous equation for TC^A and TC^B (assuming α to be the same for both); similarly $\alpha = 1.66$ and 1.63 from the equations for TC and TC^B and for TC and TC^A, respectively. All three particles appear to be equally rigid rods.

Nishihara and Doty (1958) fragmented calf skin tropocollagen, without measurable denaturation by prolonged ultrasonic irradiation, into pieces estimated by viscosity and velocity sedimentation to be one-quarter the total molecular length. They obtained

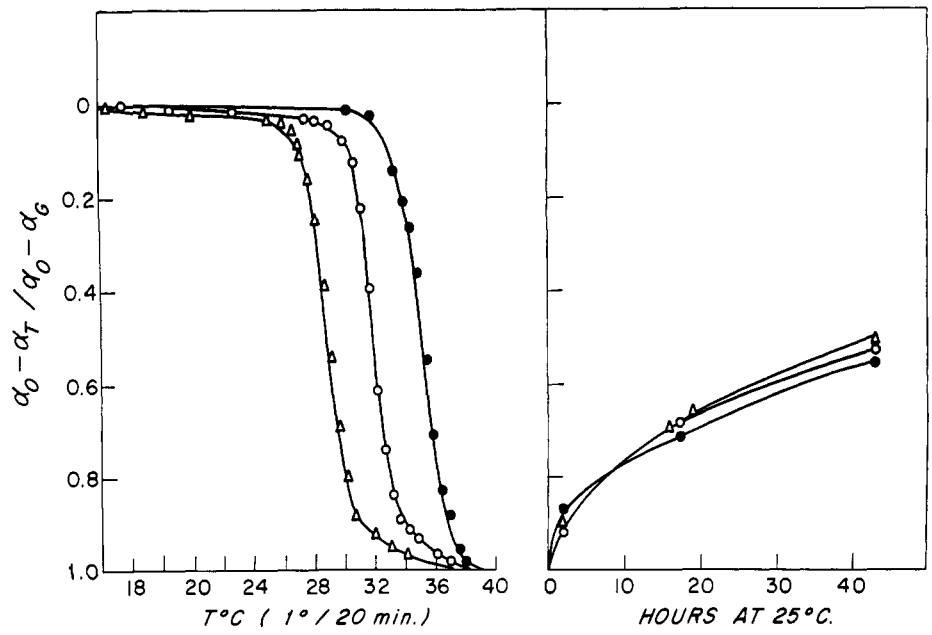

FIGURE 5: Thermal denaturation curves for isolated calf skin collagen fragments and control collagen, 0.25 M citrate, pH 3.6. Fractional change in rotation as a function of temperature. (left) Denaturation of collagen; (right) renaturation of completely denatured collagen. $\alpha^\circ = \alpha_{365}$ at 15° (start), $\alpha_T = \alpha_{365}$ at $T°$, $\alpha_G = \alpha_{365}$ at 40°. ●——●, TC; O——O, TC^A; and △——△, TC^B.

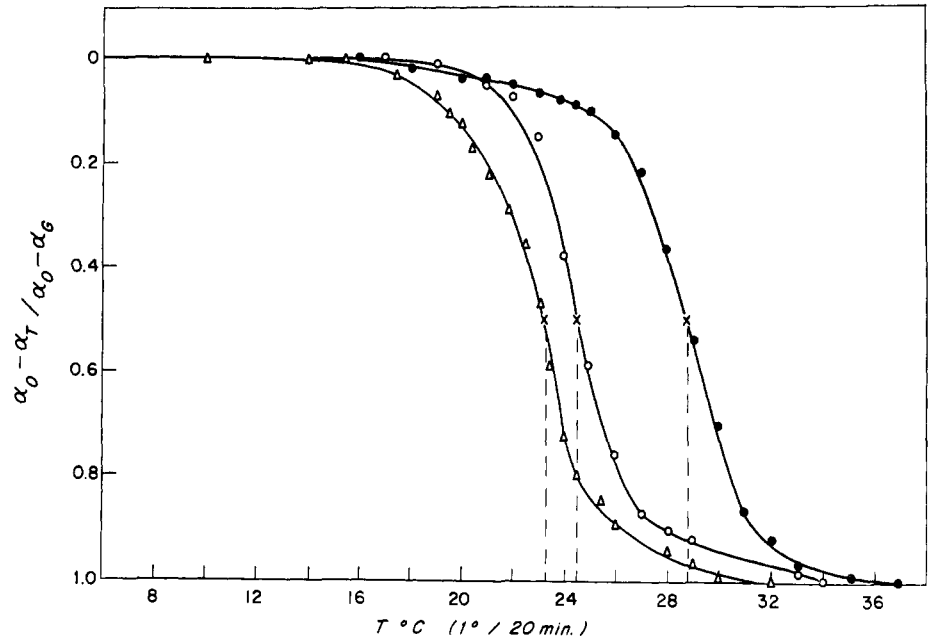

FIGURE 6: Thermal denaturation of tadpole collagen reaction products and control collagen at pH 3.0. Fractional change in rotation as a function of temperature. ●——●, TC; O——O, TC^A; and △——△, TC^B.

values of 1.4 for intrinsic viscosity, and α in the Houwink equation was estimated to be 1.80. Using their derived value $K = 1.23 \times 10^{-9}$ for sonically fragmented collagen in dilute citrate buffer we obtained values of α = 2.02, 1.86, and 1.94 for TC^B, TC^A, and TC, respectively. They also obtained a molecular weight of 105,000 for what they considered to be a homogeneous preparation of one-quarter-length fragments, in contrast to our value of 70,000 for TC^B. Hodge and Schmitt (1958) obtained one-quarter-length frag-

TABLE III: Isolated Products of Tadpole Collagenase Digested Calf Skin Collagen.

	Residues per 1000					
	(1) TC	(2) TC^A	(3) TC^B	(4)[a] $^3/_4TC^A$	(5)[a] $^1/_4TC^B$	(6)[a] (4) + (5)
3-Hypro	0.5	0	1.7	0	0.4	0.4
4-Hypro	98	103	89	77	22	99
Asp	43	43	41	32	10	42
Thr	17.8	18.5	10.8	13.9	2.7	16.6
Ser	37	34	52	26	13	39
Glu	73	73	73	54	18	72
Pro	125	122	147	92	37	129
Gly	329	330	325	247	81	328
Ala	116	126	92	94	23	117
Val	18.4	15.4	19.8	11.6	50	16.6
Met	6.4	7.0	3.0	5.3	0.8	6.1
Ile	9.2	6.9	9.7	5.2	2.4	7.6
Leu	24	22	33	16	8	24
Tyr	2.0	1.2	2.1	0.9	0.5	1.4
Phe	12.1	13.0	7.5	9.8	1.9	11.7
Hylys	8.7	9.3	11.0	7.0	2.8	9.8
NH_3	44	41	51	(31)	(13)	(44)
Lys	26	28	16	21	4	25
His	4.6	2.1	8	1.6	2.0	3.6
Arg	49	46	59	35	15	50
Recovery	999.2	1000.4	1000.6	749.5	249.5	998.8

[a] Comparison between amino acid analyses on TC with those of the sum of TC^A and TC^B.

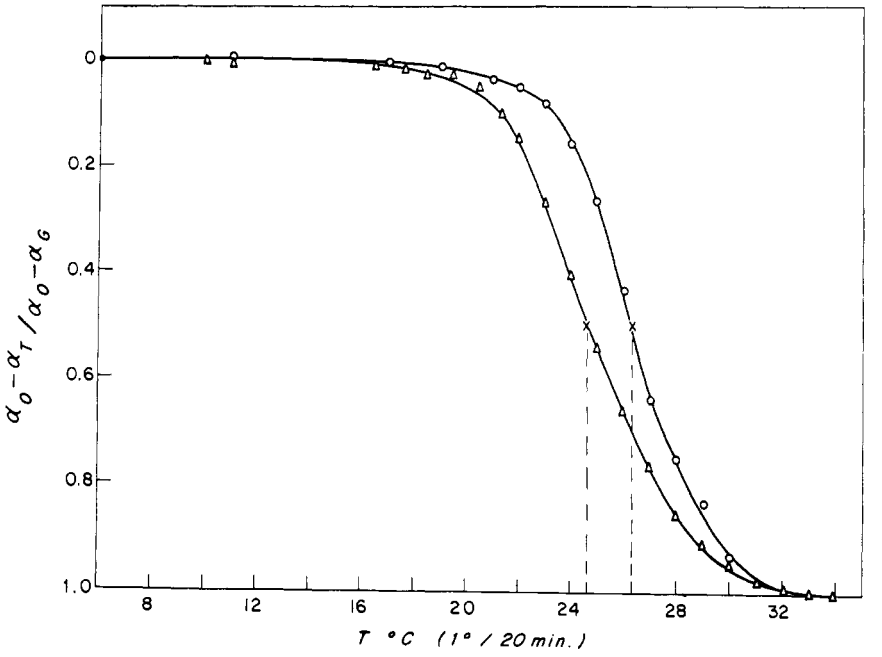

FIGURE 7: Thermal denaturation of tadpole collagen reaction products and native collagen at pH 7.6. O——O, TC^A; and △——△, TC^B.

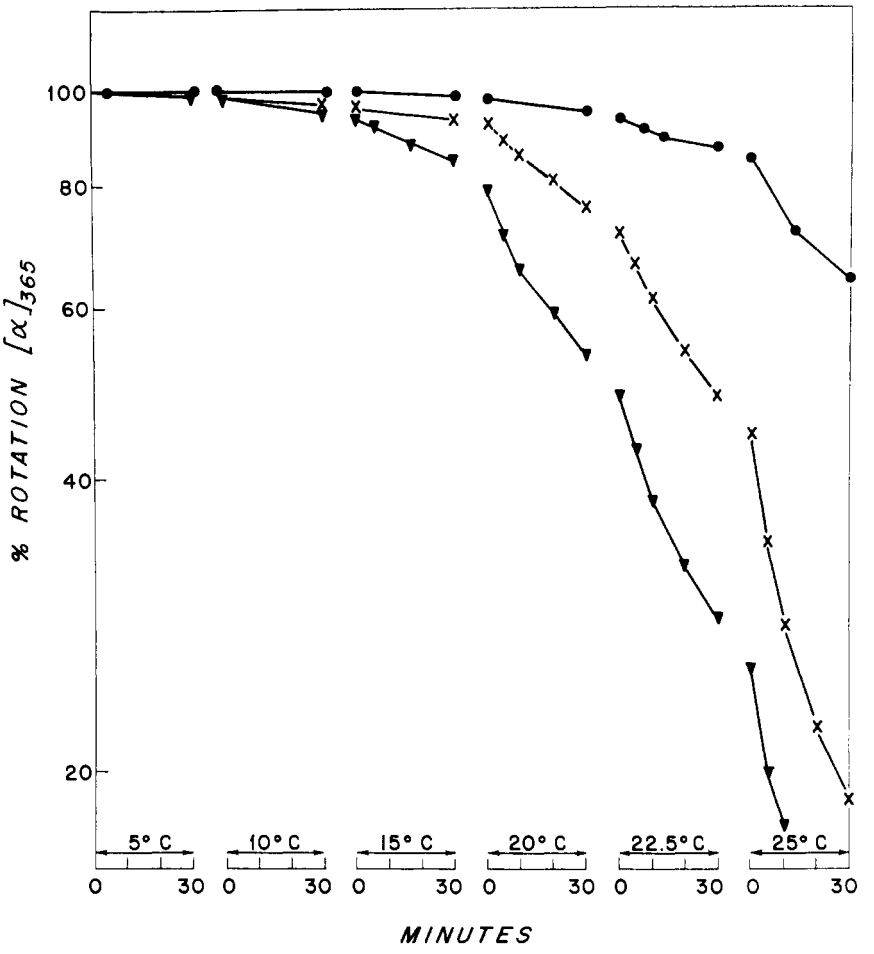

FIGURE 8: Digestion by trypsin of tadpole collagen fragments and native tropocollagen at neutral pH as a function of temperature and measured by optical rotation. Temperature elevated abruptly at 5° intervals and held for intervals of 30 min. ●——●, TC; ×——×, TC^A; and ▼——▼, TC^B.

ment SLS in electron micrographs of ATP-reconstituted collagen from the sonicated samples of Nishihara and Doty (1958). Whether the loci of scission one-quarter the molecular length from the "B" end of the molecule obtained by sonication and by enzymatic attack are identical is not yet apparent. However, if they are the same, then the three sites of breakage by sonic energy must differ in structure since they are not equally sensitive to the tadpole enzyme.

Amino Acid Analyses. The amino acid analyses of the fragments are remarkable in that the compositions are so different; only glycine, glutamic acid, and aspartic acid show little variation (Table III). All the other amino acids differ from the intact molecule by 10% or more. These data are consistent with the analyses of Kang *et al.* (1966) for the isolated randomized chains obtained from the reaction mixture. As shown in the last three columns three-quarters of the amino acid content of TC^A plus one-quarter of the content of TC^B yield values very close to those of the original tropocollagen, indicating no measurable loss of amino acids or peptides.

Conformation and Stability. Measurements of optical rotatory dispersion in the far-ultraviolet region, where the specific rotation of collagen is extremely sensitive to conformation (Blout *et al.*, 1963), indicated no loss of helical structure in the two fragments when they were compared with the native molecule (Figure 4).

It was shown previously (Gross and Nagai, 1965) that the denaturation temperature of an unfractionated reaction mixture is about 8° lower than that of native collagen. A comparison of T_m values for the isolated fragments with that of native calf skin collagen at acid pH under the conditions used by Kuhn *et al.* (1964) gave values of 36, 32, and 28.5° for TC, TC^A, and TC^B, respectively (Figure 5). The rate and extent of renaturation upon cooling to 25° for 40 hr, as shown in the right-hand portion of Figure 5, indicates that, under the conditions employed, the kinetics of renaturation are the same for all three collagen particles and about 50% total recovery of optical rotation was obtained in all three cases. The renatured preparations gave typical SLS patterns of the three types in the electron microscope after dialysis *vs.* 0.1 M acetic

acid and addition of ATP, although the amount of completely renatured material was not ascertained. It is interesting that denatured separated chains of TC^B with a molecular weight of 24,000 (Kang *et al.*, 1966) are capable of reforming the triple helix, as indicated by the production of SLS. Kuhn and Zimmerman (1966) have also been able to prepare SLS from short fragments of renatured, trypsin-degraded gelatin chains.

Comparison of shrinkage and denaturation temperatures of various collagens with amino acid composition has indicated a direct and linear relationship between stability and total imino acid content (Burge and Hynes, 1959; Piez and Gross, 1960). In contrast, in the experiments reported here, the fragment with the highest proportion of imino acids, TC^B, has the lowest denaturation temperature. It may be that stability depends, also, upon molecular length. It is also quite possible that the distribution of imino acids along the polypeptide chain may be an important factor, particularly at the ends.

The thermal denaturation curves for the isolated fragments from tadpole collagen (Figure 6) indicate the same general relationships of stability as do those of the mammalian collagen, although they melt at lower temperatures. T_m values at pH 3.0 of 28.5, 24.5, and 23.2 were obtained from TC, TC^A, and TC^B, respectively. At pH 7.6 (Figure 7) the midpoint values are shifted upward by about 1° for TC^B and 2° for TC^A. The lower denaturation temperatures are consistent with the lower imino acid content of tadpole collagen (K. A. Piez and J. Gross, unpublished).

The susceptibility of collagen fragments to tryptic digestion was tested to determine whether or not they would be more readily degraded by a typical non-collagenolytic protease. Measurements of optical rotation were made on tadpole TC^A, TC^B, and TC at neutral pH in the presence of trypsin at temperatures ranging from 5 to 25°. As shown in Figure 8, significant degradation of the helical structure begins at 10° for TC^B, 15° for TC^A, and 25° for TC. After 30 min, at 25°, TC^B has completely lost the collagen conformation and TC^A retains only about 10% whereas TC has about 70% left, showing a much slower rate of digestion in the last. The lower thermal stability of the fragments probably accounts for their susceptibility to proteolysis.

Solubility Changes. In the course of these experiments it was observed that TC^A and TC^B from mammalian collagen were readily soluble in physiologic saline at room temperature and that TC^B would also dissolve in distilled water. In addition, SLS formed from these fragments was found to dissolve completely at room temperature and re-form upon cooling. These observations are in sharp contrast with the known insolubility of native tropocollagen under these conditions.

These two characteristics of the reaction products, namely lowered thermal stability and increased solubility, point to a possible mode of collagen degradation *in vivo*. We suggest that the collagenolytic enzyme produced and secreted by the cell attacks the collagen molecules within the fibril in the extracellular space. The fibril frays and the reaction products because of their higher solubility disperse in the surrounding tissue fluid. In the dispersed state their denaturation temperatures are considerably lower than when aggregated as fibrils (Gross, 1964), and indeed lower than that of normal collagen in solution as shown above. The fragments lose their helical structure spontaneously at body temperature and become susceptible to degradation either by the collagenase, which can degrade gelatin extensively (Nagai *et al.*, 1964), or by other cellular proteases.

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Chromatography of Myosin on Diethylaminoethyl-Sephadex A-50*

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ABSTRACT: A chromatographic system using DEAE-Sephadex A-50 with 0.04 M pyrophosphate buffer, pH 7.5, and a linear KCl gradient to 0.50 M KCl separates monomeric myosin from aggregated myosin, other unidentified proteins, and ribonucleic acid (RNA). The procedure has been applied to myosin preparations from skeletal muscle of rabbit, chicken, and four species of fish.

With good preparations, adenosine triphosphatase activity across the peak varied about 20% and was greater than the starting material. As determined by sedimentation equilibrium, the molecular weight of chicken myosin purified by this procedure was found to be 5.0×10^5 g mole^{-1}.

Several investigators have employed column chromatography for the purification of myosin. Brahms (1959) and Perry (1960) used DEAE-cellulose with 0.2 M KCl buffered with Tris to pH 7.4, and obtained some degree of fractionation, but the bulk of the material passed directly through the column without retention. Perry (1960) also employed a buffer system (0.16 M KCl–0.02 M Tris, pH 8.2) with a KCl gradient that resulted in a separation of myosin from ribonucleoprotein and other proteins that passed through unretarded. However, the adenosine triphosphatase (ATPase)[1] activity varied considerably across the myosin peak, and dimers not present in the starting material were seen in the myosin purified by this procedure. Based on the work of Brahms and Brezner (1961), who showed that myosin was soluble in polyphosphates at low ionic strength, Asai (1963) used DEAE-cellulose columns, ATP in the solvent, and a KCl gradient to obtain chromatograms that were similar to those obtained with the pH 8.2 system of Perry (1960). The ATPase activity was more nearly constant across the peak, but myosin aggregates were present in the trailing part of the peak. Asai (1963) also mentioned that similar results were obtained with DEAE-cellulose and pyrophosphate as the solvent. Smoller and Fineberg (1964) observed an enhancement in ATPase activity when they purified mouse myosin by gel filtration with Sephadex G-200. Baril *et al.* (1964, 1966) used DEAE-cellulose and eluted stepwise with pyrophosphate–KCl buffers at pH 8.5 for the purification of chick myosin. They achieved separation of myosin from RNA and also from myosin–RNA complexes. The purified myosin appeared to be monomeric, but its ATPase activity was reduced.

We have developed a chromatographic procedure, with DEAE-Sephadex A-50 and KCl gradient in pyrophosphate or phosphate buffer, that separates monomeric myosin from aggregated myosins, other unidentified proteins, and RNA. This system is suitable both as an analytical tool to examine the purity of myosin preparations and as a final purification step. It should be of general utility, for similar chromatographic profiles were obtained for myosin preparations from fresh skeletal white muscle of rabbit, chicken, striped bass, skipjack, and bluefin tuna, and frozen skeletal muscle of yellowfin tuna. The ATPase activity of the peak fractions was greater than that of the starting material. The 5′-adenylic acid deaminase activity present in the original myosin was considerably re-

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[1] Abbreviation used: ATPase, adenosine triphosphatase; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphates.