Studies on the Structure of Collagen Utilizing a Collagenolytic Enzyme from Tadpole*

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ABSTRACT: Native rat skin collagen was digested with an enzyme from tadpole which is known to cleave the molecule into two unequal pieces, each of which retains the triple helical collagen structure. The larger piece (TC^A) represents 75% and the smaller piece (TC^B) 25% of the length of the molecule. Denaturation of the digestion products and fractionation by molecular sieve and carboxymethylcellulose chromatography permitted the isolation of fragments which were shown by molecular weight determination and amino acid analysis to be derived from the cleavage of the two $\alpha 1$ and one $\alpha 2$ chains each into two pieces, representing one quarter and three quarters of the original chain. The small pieces, $\alpha 1^B$ and $\alpha 2^B$, have molecular weights of 24,000. The large pieces, $\alpha 1^A$ and $\alpha 2^A$, have molecular weights of 71,000. These results demonstrate that in cleaving the collagen molecule the enzyme cleaves all three α chains in the same proportion. Therefore, the three α chains must each extend the full length of the molecule. The presence in the denatured digestion mixture of a fraction with a molecular weight twice that of the large pieces and the absence of a fraction which could be a dimer of the small pieces indicate that interchain cross-links occur only in TC^A. Since an intramolecular cross-link has been shown to be near the N-terminal ends of the α chains, these, together with the cross-link, must be at the A end of the molecule. Analysis showed that many of the amino acid residues are unequally distributed between the A and B pieces.

Recently a collagenolytic enzyme has been prepared from cultures of the back skin and tail of tadpoles which degrades native collagen in a highly selective manner under physiologic conditions (Gross and Lapiere, 1962; Lapiere and Gross, 1963; Nagai *et al.*, 1964a). Viscosity and optical rotation measurements and electron micrographs of segment long-spacing (SLS)[1] aggregates show that the enzyme cleaves the rodlike collagen molecule without loss of helical structure into two pieces (Gross and Nagai, 1965). One piece has a length approximately three quarters that of the collagen molecule. The band pattern of the SLS aggregate demonstrates that it includes the A end of the molecule. The other has a length one quarter that of the collagen molecule and comes from the B end. These pieces have been designated TC^A and TC^B. (See Figure 6 in the Discussion.) Disk electrophoresis of the denatured products also indicates a highly selective enzymatic attack. The bands corresponding to the α, β, and γ components originally present in denatured collagen disappear and are replaced by several new bands (Gross and Nagai, 1965).

It was apparent that the high selectivity and limited action of the enzyme should make it a useful tool with which to study the structure of collagen. The collagen molecule is known to contain three chains, each with a molecular weight of about 100,000, of which at least one, the $\alpha 2$ chain, is different in amino acid composition and chromatographic behavior from the other two, the $\alpha 1$ chains[2] (Piez *et al.*, 1961, 1963; Lewis and Piez, 1964a). However, it is not known how these are arranged. It has been assumed for simplicity that all three chains run the full length of the molecule, but structures in which they are folded or overlap are also possible. It is also known that there are interchain covalent cross-links in collagen which have an important physiologic role (see Harding, 1965; Bornstein and Piez, 1964; Veis and Anesey, 1965). Evidence concerning their nature and location has recently been deduced (Bornstein *et al.*, 1966).

The purpose of the present study was to isolate and characterize, after denaturation, the products of cleavage of rat skin collagen with the tadpole enzyme in an attempt to obtain information relevant to these and other aspects of collagen structure.

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[1] Abbreviation used: SLS, segment long spacing.

[2] All three α chains in codfish skin collagen have been shown to be dissimilar and have been designated $\alpha 1$, $\alpha 2$, and $\alpha 3$ (Piez, 1964, 1965). There is evidence that rat skin collagen, used in the present study, may also contain three different α chains (Bornstein and Piez, 1965). Since this is still under investigation and since the methods employed here distinguish only two kinds of α chains, the designation $\alpha 1$ will be retained for what may be a mixture.

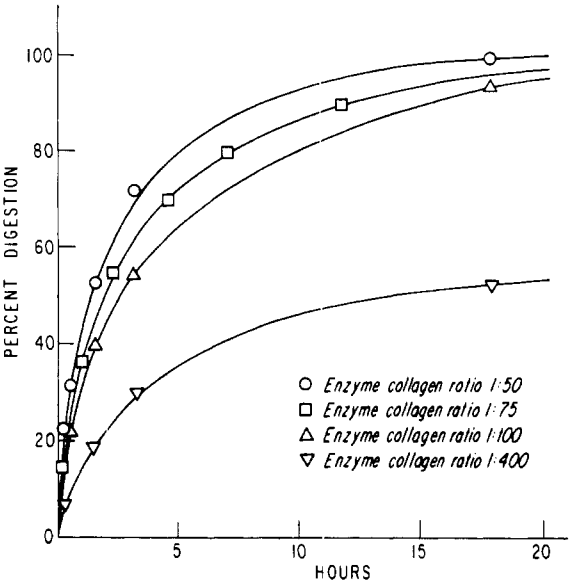

FIGURE 1: Digestion of rat skin collagen by an enzyme from tadpole as a function of enzyme:collagen ratio and time. A 60% fall in relative viscosity was taken as 100% digestion.

Methods

Preparation and Purification of Salt-Extracted Collagen. The skins from young, growing Sprague–Dawley rats were used as the source of collagen. All operations were performed below 5°. The skins were cut into small pieces, ground with a meat grinder with chips of Dry Ice, washed with a large volume of cold water overnight. The ground skin was extracted with five volumes of 0.05 M Tris buffer, pH 7.0, containing 1.0 M sodium chloride for 48 hr with occasional gentle stirring. The extracts were filtered through cheesecloth and clarified further by centrifugation at 13,000*g*. The collagen was precipitated by adding sodium chloride to a final concentration of 20%. The precipitate was collected by centrifugation and redissolved by the addition of water with stirring. Any insoluble material was separated by centrifugation, and the 20% sodium chloride precipitation was repeated. The collagen precipitate was separated by centrifugation and dissolved in 0.5% acetic acid. Dialysis was performed to reduce the salt concentration when necessary. After clarification by centrifugation, the collagen solution was dialyzed against several changes of 0.02 M dibasic sodium phosphate for 3 days. The collagen precipitate was collected by centrifugation, redissolved in 0.5% acetic acid, and precipitated again by the addition of sodium chloride to a final concentration of 5%. Precipitation from 0.5% acetic acid solution was repeated once more. Finally, the precipitate was redissolved, dialyzed exhaustively against 0.5% acetic acid, and lyophilized. The purified collagen was stored over phosphorus pentoxide at 5°. When needed, samples were dissolved by stirring overnight at 5° in the appropriate solvent and clarified by centrifugation at 100,000*g* for 3 hr.

Preparation of Tadpole Enzyme. Living, sterile tail fin and back skin strips obtained from large *Rana catesbiana* tadpoles were incubated in an amphibian balanced cold solution and the crude enzyme was harvested 3 days later from the incubation medium. The powder obtained from the dialyzed and lyophilized fluid was fractionated by differential ammonium sulfate precipitation and molecular sieve chromatography to be described in detail by Nagai, Lapiere, and Gross (in preparation). The semi-purified enzyme was stored in neutral solution at −20°.

Digestion of Collagen by Tadpole Enzyme. Digestion was performed at 20 ± 0.01° in 0.05 M Tris buffer, pH 7.5, containing 0.2 M sodium chloride and 0.005 M calcium chloride. The enzyme:collagen weight ratio ranged from 1:400 to 1:50 and the final concentration of collagen was 1–2 mg/ml. The weight of enzyme was approximated from the absorbance at 280 mμ using an extinction coefficient of 10 ($E^{1\%}_{1\,cm}$). The course of the reaction was followed by viscometry and the degree of digestion was confirmed by acrylamide gel electrophoresis. The reaction was stopped by acidifying the reaction mixture with 1.0 N acetic acid, and the enzyme was irreversibly inactivated by heating the mixture to 60° for 10 min. A collagen solution, treated in an identical manner with the enzyme omitted, served as control.

Viscometry. Ostwald viscometers with a flow time for water of about 90 sec at 20° were used. At zero time, the desired amount of tadpole enzyme and an identical volume of Tris buffer were added to the experimental and control collagen solutions, respectively. After thorough mixing, an aliquot from each was transferred to a viscometer and the relative viscosity was determined periodically. When digestion was complete at 20°, there was a 60% reduction in relative viscosity.

Acrylamide Gel Electrophoresis. This technique, as modified for collagen components by Nagai *et al.* (1964b), allowed the analytical separation by molecular weight of α, β, and γ components as well as the fragments resulting from the action of the tadpole enzyme on native collagen. A 10 or 12.5% running gel was used in these studies because the smaller fragments migrated at the buffer front in the usual 5% gel.

Molecular Sieve Chromatography. After heat inactivation of tadpole enzyme, the reaction mixture was dialyzed against 0.5% acetic acid at 5° to remove salts and lyophilized. About 50 mg of the product was dissolved in 7 ml of 1.0 M calcium chloride, adjusted to pH 7.0, warmed briefly to 40° to destroy any aggregates that might have been formed, and chromatographed on a 2.4 × 100 cm Sephadex G-200 column at room temperature. Approximately 10% cellulose (wet volume) was mixed with the Sephadex. Appropriate effluent fractions were pooled and protein was isolated by desalting on Sephadex G-25 using a pyridine acetate buffer followed by lyophilization (Piez *et al.*, 1963).

Carboxymethylcellulose Chromatography. The fractions from Sephadex G-200 chromatography were further fractionated by carboxymethyl- (CM-) cellulose chromatography. The procedure was similar to that previously described for intact α chains (Piez *et al.*,

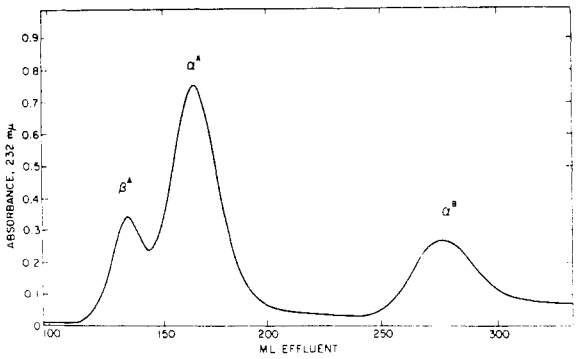

FIGURE 2: Molecular sieve chromatography of approximately 60 mg of rat skin collagen digested to completion with an enzyme from tadpole. Conditions: Sephadex G-200 containing 10% cellulose, 1.0 M calcium chloride, pH 7.0, 15 ml/hr, room temperature.

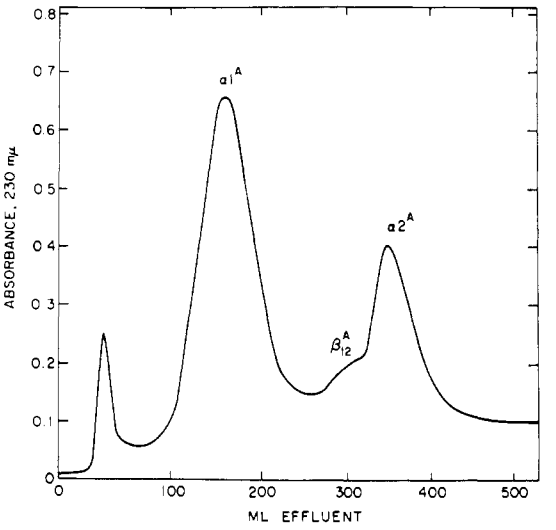

FIGURE 3: CM-cellulose chromatography of approximately 50 mg of α^A isolated by molecular sieve chromatography. The small amount of $\beta^A{}_{12}$ resulted from incomplete resolution of α^A and β^A (Figure 2). See text for conditions.

1963). The column, 18 × 150 mm, was eluted at the rate of 200 ml/hr. The buffer used for chromatography of the larger fragments was 0.05 ionic strength sodium acetate, pH 4.8, on which was superimposed a linear gradient from 0.0 to 0.11 M sodium chloride over a total volume of 800 ml. The buffer used for chromatography of the smaller fragments was 0.03 ionic strength sodium acetate, pH 4.8, with a superimposed linear gradient of 0.0–0.14 M sodium chloride over a total volume of 800 ml. The desired protein fractions were pooled, desalted on Sephadex G-25 in pH 4.8 pyridine acetate buffer, and lyophilized.

Molecular Weight Determinations. The molecular weights of the fragments isolated by Sephadex G-200 and CM-cellulose chromatography were measured at 25° in 1 M calcium chloride, pH 7.0, by the high-speed sedimentation equilibrium method of Yphantis (1964). The experimental procedures and methods of measurement and calculation were the same as those described for molecular weight determination of the α chains from codfish skin collagen (Piez, 1965). Molecular weights were expressed as M_w for the whole cell, M_w at zero concentration ($y = 0$), and M_z (M_w at the bottom of the cell). The partial specific volume, 0.705, previously used for denatured rat skin collagen α chains in pH 4.8 acetate (Lewis and Piez, 1964b) was also used here. If the value of 0.695, measured for denatured codfish skin collagen in 1 M calcium chloride (Piez, 1965), had been used, the results would be about 4% lower.

Amino Acid Analysis. Protein samples were hydrolyzed in 6 N HCl for 24 hr at 108° in tubes sealed under nitrogen, and the amino acid composition was determined on an automatic amino acid analyzer. Corrections were made for decomposition of threonine, serine, methionine, and tyrosine and incomplete release of valine as previously described (Piez *et al.*, 1960).

Results

Course of Digestion. The course of digestion of rat skin collagen by tadpole enzyme at various enzyme: collagen ratios is shown in Figure 1. At a ratio of 1:50, digestion was nearly complete in 10 hr, while at a ratio of 1:400 the digestion was only 50% complete at the end of 24 hr. For the experiments described below, two sets of conditions were used. Complete digestion was achieved by using an enzyme:collagen ratio of 1:50 for 16 hr. Partial digestion (40%) was obtained at a ratio of 1:400 for 6 hr. Digestion of a glycine-^{14}C labeled collagen in a dialysis sack under the latter conditions and analysis of the dialysate for radioactivity demonstrated that less than 0.2% of the glycine in the collagen sample became dialyzable.

Molecular Sieve Chromatography. Collagen which was completely digested by enzyme and chromatographed in the denatured form on Sephadex G-200 showed three peaks (Figure 2) which appeared homogeneous except for some tailing of the last peak. Molecular weights were determined on protein in fractions taken from each peak. Values of approximately 150,000, 71,000, and 24,000 were obtained (see the detailed discussion below). Measurements of the areas under the peaks showed that the 24,000 molecular weight protein accounted for 25% of the total protein, assuming identical extinction coefficients. Based on the fact that 24,000 is 25% and 71,000 is 75% of the molecular weight of the original α chains (about 95,000), the peaks were identified tentatively and later confirmed to be as follows (see below). The 24,000 molecular weight protein represents fragments derived from portions of the three α chains originally present in TC^B and is designated α^B. The 71,000 molecular weight protein represents the remainder of the α chains originally present in TC^A and is designated α^A. The 150,000 molecular weight protein must be derived from cross-linked chains (β components) and since this fraction has a molecular weight

TABLE I: Molecular Weights of Rat Skin Collagen α Chains and Tadpole Enzyme Digestion Products after Denaturation.

Component	Approx Concn (mg/ml)	Speed (rpm)	M_w Whole Cell	M_w At $y = 0$	M_z[a]
$\alpha 1$	0.24	21,740	95,600	96,100	94,300
$\alpha 1$[b]	0.12	21,740	93,200	92,500	95,000
$\alpha 2$	0.12	21,740	119,300	94,500	172,900
α^A	0.10	24,630	69,500	65,000	82,300
α^A[b]	0.05	24,630	71,000	75,100	66,000
$\alpha 1^A$	0.20	24,630	73,300	72,000	73,500
$\alpha 2^A$	0.07	24,630	71,100	65,100	82,300
α^B	0.20	37,020	22,500	21,500	25,000
α^B[b]	0.10	37,020	26,000	27,200	24,600
β^A	0.10	19,160	150,400	131,100	189,800

[a] M_w extrapolated to cell bottom. [b] See Figure 5 for more complete data.

approximately twice that of α^A, it must have originally been present in TC^A and is designated β^A. The amount of β^A was consistent with the measured content of β component, about 20%, in the salt-extracted collagen used. There was no evidence in any experiments for the presence of a dimer of α^B, demonstrating that the cross-link was always in TC^A and never in TC^B.

CM-Cellulose Chromatography. The α^A fraction, isolated by Sephadex G-200 chromatography, was chromatographed on a CM-cellulose column, using an ionic strength gradient of 0.05–0.16. An example of the separation achieved is illustrated in Figure 3. The similarity of the peaks to those of α chains from undigested collagen was obvious, and they were designated $\alpha 1^A$ and $\alpha 2^A$ by analogy. The ratio of $\alpha 1^A$:$\alpha 2^A$ was close to 2.0 as would be expected since $\alpha 1$ and $\alpha 2$ occur in this ratio (Piez *et al.*, 1963). These identifications were confirmed by amino acid analysis (see below). A small shoulder preceding $\alpha 2^A$ was present in these chromatograms. This was presumably $\beta^A{}_{12}$ arising from incomplete resolution of α^A and β^A on Sephadex G-200 (Figure 1). It was not studied further and no attempt was made to chromatograph the β^A fraction.

When the α^B fraction isolated from completely digested collagen was chromatographed on CM-cellulose, a complex pattern containing many peaks was obtained. It was felt that the initial cleavage may have been followed by additional proteolysis producing altered fragments. This may have occurred near the initial point of attack due to a "loosening" of the helical structure. This conclusion was also consistent with the slight asymmetry of the α^B fraction on Sephadex G-200 (Figure 2). Therefore, α^B was isolated from partially digested collagen in an attempt to avoid this problem. Under these conditions, two major peaks were obtained which were identified as $\alpha 1^B$ and $\alpha 2^B$ by chromatographic behavior and amino acid analysis (see below). The chromatograms (Figure 4) showed a number of minor components, indicating that additional proteolysis had not been completely prevented. Amino acid analysis of the protein obtained from these peaks indicated that they were closely related to either $\alpha 1^B$ or $\alpha 2^B$. The loss of only a few amino acids could have been involved since, as noted above, only trace amounts of the collagen became dialyzable during digestion.

All of the CM-cellulose chromatograms show a forepeak of varying size. It is presumed to arise from nonspecific degradation since the peak is not reproducible in size and it recurs when protein components isolated from the effluent are rechromatographed. Nonprotein ultraviolet-absorbing contaminants may also contribute to this peak.

Molecular Weights. Molecular weights were determined for $\alpha 1$ and $\alpha 2$ chains from rat skin and for the digestion products after chromatography on Sephadex G-200 and CM-cellulose (Table I) using the high-speed sedimentation equilibrium method of Yphantis (1964). As in the case of codfish skin collagen α chains (Piez, 1965), the best results were obtained when a portion of the effluent from a chromatogram was dialyzed against 1 M calcium chloride at pH 7.0 and used for molecular weight measurements without isolating protein. Except for $\alpha 2$ and β^A all samples showed good homogeneity. Typical data for several samples showing the molecular weight distribution across the centrifuge cell are shown in Figure 5. In the case of $\alpha 2$, contamination with β_{12} would be expected since resolution is not complete (Piez *et al.*, 1963; Lewis and Piez, 1964) and accounts for the high value for M_z (Table I). The value of M_w (at $y = 0$), 94,500, should be close to the monomer molecular weight. This value and the values obtained for $\alpha 1$, which averaged about 95,000, are in good agreement with results obtained previously for α chains from rat skin and codfish skin collagens (Lewis and Piez, 1964; Piez, 1965). The double-chain component β^A would be expected to be contaminated with higher aggregates

TABLE II: Amino Acid Composition of the Fragments from the Digestion of Rat Skin Collagen by an Enzyme from Tadpole.

	Residues/Mole[a]							
Amino Acid	$\alpha 1^A$	$\alpha 1^B$	Total	$\alpha 1$ (found)	$\alpha 2^A$	$\alpha 2^B$	Total	$\alpha 2$ (found)
3-Hydroxyproline	0	1 (0.6)	1	1 (0.7)	0	0	0	0
4-Hydroxyproline	75	23	98	101	64	20	84	84
Aspartic acid	37	12	49	48	33	15	48	48
Threonine	15	5 (4.6)	20	20	18	2 (1.8)	20	20
Serine	21	15	36	40	30	12	42	39
Glutamic acid	59	20	79	78	58	15	73	72
Proline	92	36	128	130	83	29	112	114
Glycine	264	87	351	348	259	87	346	345
Alanine	96	23	119	118	86	23	109	108
Valine	19	3 (3.4)	22	22	26	11	37	38
Methionine	6 (5.6)	1 (1.4)	7	7 (7.1)	4 (4.3)	0 (0.1)	4	5 (4.7)
Isoleucine	3 (3.1)	5 (4.7)	8	7 (6.9)	14	6 (6.0)	20	20
Leucine	13	6 (6.2)	19	19	23	12	35	37
Tyrosine	1 (0.6)	1 (0.9)	2	2 (2.2)	1 (0.3)	2 (2.0)	3	3 (3.5)
Phenylalanine	10 (9.8)	2 (2.2)	12	12	10 (9.9)	1 (1.0)	11	12
Hydroxylysine	3 (3.3)	2 (2.4)	5	6 (5.7)	7 (7.3)	3 (3.3)	10	10 (9.8)
Lysine	27	5 (4.7)	32	31	19	4 (4.0)	23	24
Histidine	1 (1.2)	1 (1.2)	2	2 (2.1)	3 (3.3)	6 (6.1)	9	9 (9.2)
Arginine	37	16	53	52	41	14	55	54

[a] Assuming the following molecular weights: $\alpha 1$ and $\alpha 2$, 95,000; $\alpha 1^A$ and $\alpha 2^A$, 71,000; $\alpha 1^B$ and $\alpha 2^B$, 24,000. Where the number of residues is 10 or less, the actual value obtained is shown in parentheses.

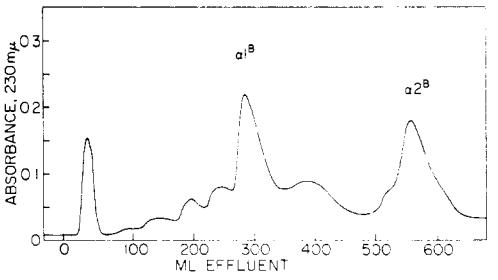

FIGURE 4: CM-cellulose chromatography of approximately 20 mg of α^B isolated by molecular sieve chromatography of collagen partially digested by an enzyme from tadpole. The minor components presumably arose as a result of further digestion of $\alpha 1^B$ and $\alpha 2^B$ following the initial cleavage. See text for conditions.

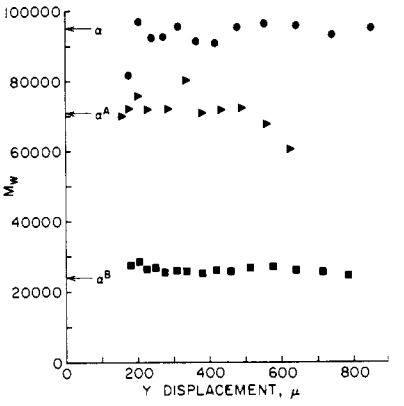

FIGURE 5: Molecular weight data from high-speed sedimentation equilibrium analysis of α chains (●) and the fragments α^A (▶) and α^B (■) resulting from digestion with an enzyme from tadpole. The arrows indicate the average values from several experiments. See text and Table I for conditions.

which are usually present in collagen samples (for example, γ component might yield a fragment, γ^A, with a molecular weight over 200,000) and also with α^A since resolution is not complete (Figure 2). This would account for the heterogeneity observed (Table I). However, the results are consistent with the identification of the major part of the protein in β^A as the dimer of α^A. Similar molecular weights, averaging 71,000, were obtained for α^A, $\alpha 1^A$, and $\alpha 2^A$. The molecular weight of α^B was taken to be 24,000. Molecular weights were not obtained for $\alpha 1^B$ and $\alpha 2^B$ but they should be similar since α^B was homogeneous.

Amino Acid Composition. Amino acid analysis of the fragments derived from $\alpha 1$ and $\alpha 2$ and isolated by

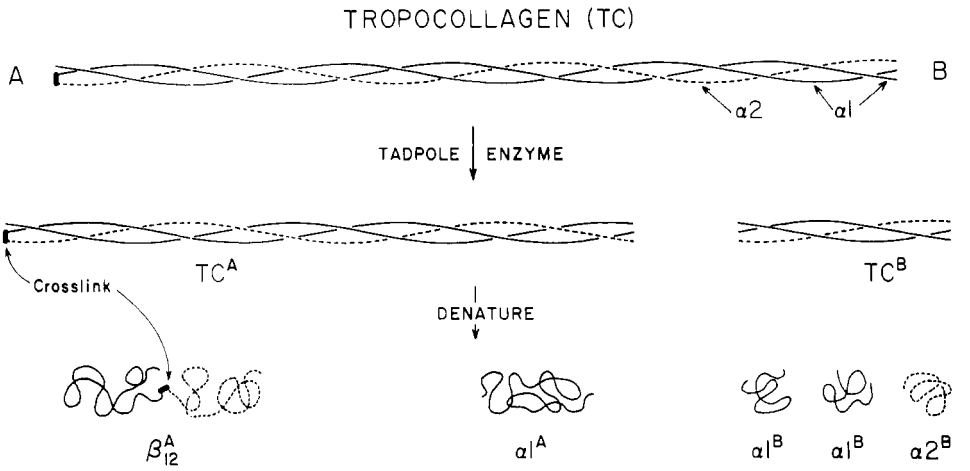

FIGURE 6: Diagrammatic representation of the collagen molecule and the pieces resulting from digestion with an enzyme from tadpole. The minor helix of the individual chains is not shown; the major helix is not to scale. The letters A and B refer to the designations employed in electron microscopy to distinguish the ends of the molecule. In a sample of extractable collagen some molecules would be cross-linked between $\alpha 1$ and $\alpha 2$ as shown, others between the two $\alpha 1$ chains, and some not at all. The denatured products would then include $\alpha 2^{A}$ and β^{A}_{11} in addition to those shown.

CM-cellulose chromatography confirmed the identifications indicated in Figures 3 and 4. The results, expressed as residues/mole using the measured molecular weights, are shown in Table II. The sums of the residues in $\alpha 1^{A}$ and $\alpha 1^{B}$ and in $\alpha 2^{A}$ and $\alpha 2^{B}$ agree very closely with the residue found in intact $\alpha 1$ and $\alpha 2$. Every amino acid residue was accounted for within experimental error.

Discussion

Characterization of the fragments derived from denaturation of collagen treated with tadpole enzyme demonstrates that each of the three α chains is cleaved into two pieces representing one quarter and three quarters of the original chains. Since the native molecule is cleaved in the same proportion, it can be concluded that all three α chains extend the full length of the molecule. If there were an overlap or folding involving more than a few per cent of the length of the α chains, the corresponding pieces from $\alpha 1$ and $\alpha 2$ would have different sizes. The indicated structure is shown diagrammatically in Figure 6.

A clear result was also obtained with regard to the position of the cross-link. The presence of dimers of α^{A} in an amount sufficient to account for the proportion of cross-linked components in the original sample and the complete absence of dimers of α^{B} demonstrate that cross-links occur only in TC^{A}. The evidence presented here places the cross-link no closer than somewhere in TC^{A}. Evidence from other experiments (Bornstein *et al.*, 1966) places it very near the N-terminal ends of the α chains. Since the α chains extend the full length of the molecule, the N-terminal ends and the cross-link must be at the A end of the molecule as shown in Figure 6.

The data in Table II show that the distribution of amino acids in the A and B portions of the molecule is unequal in certain respects. If they are divided into types (acidic, basic, imino, hydroxy, and nonpolar), it can be seen that the average character of the pieces cannot be greatly different from that of the parent chain. The only exception to this is a higher concentration of hydroxyamino acids in $\alpha 1^{B}$ compared to $\alpha 1^{A}$. However, individual amino acids show large differences in concentration. For example, in $\alpha 1$ five of the eight isoleucines and two of the five hydroxylysines but only three of the twenty-two valines and one of the seven methionines are in the small piece; in $\alpha 2$ two of the three tyrosines and six of the nine histidines but only one of the eleven phenylalanines and none of the four or five methionines are in the smaller piece. Other unequal distributions are present. These results indicate significant differences in detail between various regions of the molecule and are inconsistent with any model in which the α chains are composed of linear arrays of identical subunits (Petrushka and Hodge, 1963). A similar conclusion has been reached by cleavage of methionyl bones in collagen α chains and analysis of the resulting peptides (Bornstein and Piez, 1965). The evidence does not rule out the possibility of nonidentical intrachain subunits.

It might be expected that the amino acids present in small amounts in $\alpha 2^{B}$ and $\alpha 1^{B}$ would be found in amounts close to whole numbers since these fragments are relatively small. This is the case for $\alpha 2^{B}$, but of the eleven amino acids in $\alpha 2^{B}$ present at levels below 10 residues/mole, six were found in amounts sufficiently different from whole numbers to question the validity of rounding off the values (Table II). Although this could be experimental error, it is also consistent with the suggestion (Piez, 1964; Bornstein and Piez, 1965)[2]

that the two $\alpha 1$ chains are not identical; that is, $\alpha 1^B$ (and therefore $\alpha 1^A$ also) may be a mixture of two components with different amino acid compositions.

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On the Molecular Weight of Myosin. II*

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ABSTRACT: The number-average molecular weight of myosin, measured osmometrically, has been found to be *ca.* 4.7×10^5 g mole^{-1}. The weight-average molecular weight, inferred from the equilibrium distribution when the same preparations were in an ultracentrifugal field, was found to be 4.8×10^5 g mole^{-1} using midpoint analysis and 5.1×10^5 g mole^{-1} using whole column analysis.

These values appear to be consistent with chemical determinations of minimum molecular weight. It is speculated that molecular weight estimates of the order of 6×10^5 g mole^{-1} are characteristic of preparations which have been exposed to molar concentrations of salts.

In order to consider the substructure and enzymatic properties of myosin, it is necessary to know its molecular weight, with an uncertainty of 20% or less. This is not easy, partly because the myosin system is sensitive to conditions imposed during preparation, and partly because the molecular weight is high. Excepting the early report by Portzehl *et al.* (1950), the many physical estimates now available are weight-average molecular weights ($\bar{M}_w$), usually obtained by sedimentation, sometimes by light scattering. Because osmometric and preparative techniques have greatly improved since 1950, it seemed opportune to attempt again to measure the number-average molecular weight ($\bar{M}_n$) of myosin. On the same preparations used for osmometry we have also measured $\bar{M}_w$ by sedimentation to equilibrium, and we have listed molecular weight estimates based on chemical stoichiometry toward substrates and modifiers of myosin adenosine triphosphatase (ATPase).[1] All data taken together assure that the average molecular weight of rabbit skeletal myosin as used in most laboratories is less than 5×10^5 g mole^{-1}, very probably $4.6–4.8 \times 10^5$ g mole^{-1}. Because

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[1] Abbreviations used: ATPase, adenosine triphosphatase.