Biochemistry

 Volume 4, Number 2 February 11, 1965

Effects of Pepsin Treatment on the Interaction Properties of Tropocollagen Macromolecules*

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ABSTRACT: A procedure for obtaining purified soluble tropocollagen fractions from calfskin is described. These preparations are suitable for studying the composition, structure, and interaction properties of the macromolecules.

The action of pepsin on such tropocollagen solutions results in the release of dialyzable peptides with an amino acid composition markedly different from that of the bulk tropocollagen. Concomitantly with the release of peptides, pepsin breaks intramolecular cross-links and alters both the antigenic and the aggregative properties of the tropocollagen molecule, but no changes are discernible by electron microscopic examination.

Collagen is a structural protein of great biological significance. It is present in mammals in concentration higher than that of any other protein and serves primarily a mechanical function. Study of its detailed structure and composition is rewarding not only because of its abundance and obvious physiological importance and because it represents a class segregated early in evolution and separate from other protein classes structurally, but also because such study may throw light upon the molecular correlates of some aspects of aging processes and of certain diseases, particularly the rheumatoid and cardiovascular diseases. Before molecular changes in the molecules in pathological states can be fruitfully studied, the range of variation occurring during normal development must be known (Schmitt, 1960).

Advances made in this laboratory during the last decade have been recently reviewed (Schmitt and Hodge, 1960; Schmitt, 1963, 1964). The native collagen fiber is built up by a highly ordered process of linear and lateral aggregation of thin, highly elongate macromolecules called *tropocollagen.* With a diameter of about 14 A, a length of 2800 A, and a molecular weight of about 300,000, tropocollagen macromolecules are composed of three hydrogen-bonded chains intertwined in a characteristic helical configuration to produce stiff rods in the native state. Aggregation of tropocollagen in a polarized quarter-stagger array produces *collagen* fibrils with a fundamental axial periodicity of about 700 A. "Collagen" thus represents a particular aggregation state of constituent tropocollagen molecules which themselves manifest small but important changes in intramolecular structure and in intermolecular bonding. These modifications probably occur from the time the tropocollagen is synthesized until the soluble molecules are firmly bonded to form the mature collagen fibers. Studies of the type herein

* From the Department of Biology, Massachusetts Institute of Technology, Cambridge. *Received October 9, 1964.* This investigation was supported by grants from the National Institute of Neurological Diseases and Blindness (NB-00024) and the National Heart Institute (HE-08763) of the National Institutes of Health, U.S. Public Health Service. A preliminary report of this investigation was published by Rubin *et al.* (1963).

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described are intended to illuminate the nature of these physiologically significant changes.

Protruding from the triple-helix body of native tropocollagen are peptides having composition different from that of the triple-helix portions. These peptides are removed (to a degree dictated by the enzyme specificity) by the action of proteases, and with their removal intramolecular interchain bonds are broken. Because the formation of fibrous aggregates of tropocollagen is also considerably changed, it was concluded that one or more of the peptides must be located at near-terminal positions; they were therefore termed *telopeptides* (Rubin *et al.*, 1963). It was subsequently shown that, like the protease attack on native tropocollagen, the antigenic response to injected heterologous tropocollagen is directed against the peptide appendages external to the triple helix (Schmitt *et al.*, 1964). This finding adds weight to the suggestion that the peptides may participate not only in the biological control of fibrogenesis through alterations of intra- and intermolecular bonding, but also in pathological alterations in the so-called "collagen diseases" of the autoimmune type (Rubin *et al.*, 1963).

The conclusions briefly mentioned did not enjoy immediate acceptance by other workers in the field probably primarily because of differences in the nature and the purity of the tropocollagen prepared in each laboratory. Since the peptides scissionable by protease (other than collagenase) from tropocollagen represent but a few per cent of the total mass, it is evident that the peptides can be studied properly only in the purest tropocollagen preparations. Assessment of the purity of each investigator's starting material cannot be made unless the analytical data are published. An important purpose of the present communication is to provide not only the analytical data on which the evidence for the existence of the telopeptides rests, but also the method of preparation of the purified tropocollagen. A recent convention (Kohn *et al.*, 1964) achieved a consensus regarding terminology of intramolecular variants of tropocollagen. Hopefully a consensus may also soon be achieved regarding standard starting material for investigations of the composition, structure, and properties of tropocollagen.

The purified tropocollagen described herein was used to study the action of noncollagenase proteases. The action of pepsin is described in this communication; that of pronase, trypsin, chymotrypsin, and elastase (M. P. Drake, P. F. Davison, S. Bump, A. L. Rubin, and F. O. Schmitt, paper in preparation) and further studies of antigenicity and interchain bonding sites will be published elsewhere.

Materials and Methods

Reagents. Inorganic reagents were commercial reagent grade. Water was double distilled. Pepsin was a twice-crystallized salt-free product of the Worthington Biochemical Corp.

Analytical Methods. Phosphorus determinations were made by the method of Martland and Robison (1926), nitrogen determinations by a micro-Kjeldahl method (Markham, 1942), hexose by the anthrone method of Seifter *et al.* (1950), hexosamines by the method of Boas (1953), tyrosine by the method of Bernhart (1938), and hydroxyproline by the method of Martin and Axelrod (1953). Amino acid analyses were performed on an automatic analyzer (Technicon Instruments Corp.). Pepsin activity was assayed by the procedure of Anson (1939) with the use of hemoglobin as a substrate.

Free-flow electrophoresis was performed on the apparatus (free-flowing continuous electrophoretic separator, Bender and Hobein, Model FF) designed by Hannig (1961). Tropocollagen at 0.05% concentration in an appropriate acetic-pyridine or *p*H 2.9 0.02 M citrate buffer was injected into the chamber; the traverse time was 30 minutes. A lateral field of approximately 40 v cm^{-1} was maintained. Analytical moving-boundary electrophoresis was performed in a Perkin-Elmer apparatus.

Viscosity measurements were made in Ubbelohde single-bulb and multibulb viscometers in a 20° (± 0.01°) water bath. The progressive changes in viscosity during dialysis against water were measured in a special viscometer assembly (Altgelt).[1] Optical rotation measurements were made on a Rudolf Model 80 photoelectric polarimeter with an oscillating polarizer prism; a mercury arc was used as a light source with a Bausch and Lomb monochromator to select the 365-mμ spectral line; ultracentrifugation was performed on a Spinco Model E instrument, and electron microscopy on a Siemens Elmiskop 1. Radioactivity of ^{131}I was measured on Tracerlab automatic counting equipment.

Treatment of Tropocollagen with Pepsin. Solutions of tropocollagen (*ca.* 0.2%) and of 0.1% pepsin in 0.05% acetic acid were dialyzed for 24–48 hours versus several changes of 0.05% (*p*H 3.5) acetic acid. A suitable aliquot of the pepsin solution was then added to 100 ml of the tropocollagen solution to make the weight ratio of pepsin-collagen 1:100. After mixing, the pepsin-collagen solution was dialyzed versus 500 ml of a fresh solution of 0.05% acetic acid on a shaker at 20° for 24 hours. The dialysis was then continued at 4° for an additional 48 hours. The dialysate was concentrated to dryness on a Buchler vacuum rotating evaporator (using a 55° bath). In early experiments the collagen and the pepsin in the dialysant were clearly separated by free-flow preparative electrophoresis, and the pepsin activity in the separated pepsin peak accounted for all of the pepsin used. Subsequently a simpler method was used to remove pepsin. The dialysant was dialyzed versus 15% KCl–0.02 M Na_2HPO_4 to precipitate the collagen and to denature and separate the pepsin. After the harvested collagen was redissolved in 0.05% acetic acid and reprecipitated in the KCl-PO_4 solution twice more, the pepsin was virtually eliminated.

[1] The viscometer used for the water dialysis experiments was designed and constructed in these laboratories by Dr. K. Altgelt. Present address: Richmond Laboratories, Standard Oil of California, Richmond, Calif.

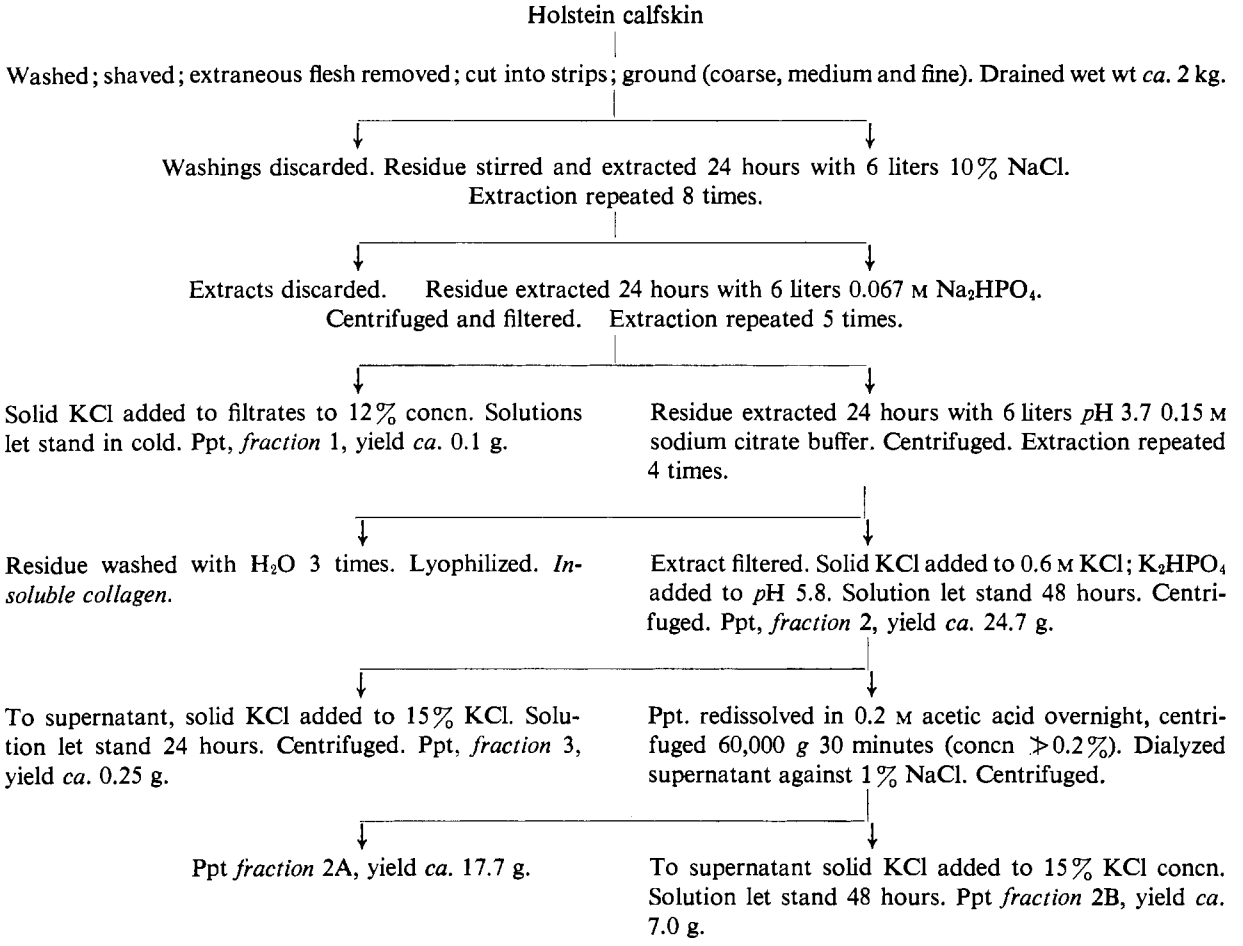

FIGURE 1: Flow sheet for the preparation of J4 calfskin tropocollagen.

Iodination. Solutions (2%) of bovine serum albumin and bovine glycoprotein were iodinated at a specific activity of 100 μc/mg iodine by the method of Gilmore *et al.* (1954), with the omission of guanidine hydrochloride.

Hydrolysis. Two ml of glass-redistilled constant-boiling HCl and 1 μl of redistilled phenol (saturated with water) were added to a sample (1–5 mg) of dry collagen in an ampoule and the solution was flushed with N_2 three times before sealing. The flushing was accomplished by alternately subjecting the contents to a vacuum, then breaking the vacuum with N_2. The sealed ampoules were placed in a temperature-controlled oven at 102° for the desired hydrolysis period of 20, 40, 70, or 140 hours. The addition of phenol, as suggested by Sanger and Thompson (1963), eliminated a varying loss of tyrosine noted in early analyses.

Results

Preparation of Calfskin Tropocollagen and Criteria of Purity. Because the development of suitable purification processes shares significance with studies of protease action, the report on purification is presented in the experimental rather than in the methods section of this paper.

The isolation and purification of the tropocollagen is described in the flow sheet in Figure 1. The yields shown for the various fractions refer to dry weights. Immediately after the calves were slaughtered, the skins were frozen with dry ice and stored frozen. Thawing was accomplished in running cold water, and refrigeration of the material was maintained throughout the preparation.

The steps in Figure 1 consist of extraction, precipitation, and redissolution of the tropocollagen under selective conditions; these steps were continued until certain parameters (e.g., optical rotation, and glycine and hydroxyproline contents) were maximized with respect to the nitrogen content of the solution. With progressive purification, the content of tyrosine, methionine, hexose, and hexosamine was found to fall.

Large-batch preparations of tropocollagen were made so that repeated experiments could be done on the same starting material. The average elapsed time in the preparation was 8–10 weeks. The purified salt-precipitated fractions were stored frozen, and were redissolved as required.

TABLE I: Coprecipitation of Other Proteins with Tropocollagen.

Protein Added		Conditions of Precipitation (cpm)[a] KCl	NaCl	K_2HPO_4
Albumin	Supernatant	1,800	13,000	7,600
	Precipitate	10,000	220	10,700
Glycoprotein	Supernatant	14,700	16,600	15,700
	Precipitate	2,100	230	3,300

[a] The counts in the precipitate are corrected for the occluded volume of supernatant.

The time during which the skin extracts were allowed to stand in acetic acid was kept to a minimum—less than 1 week, *in toto*.

Five different calfskin preparations (J2 to J6) were used for these experiments, of which J4 to J6 were prepared following Figure 1. Preparations J2 and J3 differed only in that they were allowed to stand in acidic solutions longer.

The desirability for minimizing exposure to low *p*H was evident when it was noted that progressive changes in viscosity occurred in acidic tropocollagen solutions with time. These changes, which occurred relatively slowly in the case of acetic acid but much more rapidly in citrate, were eventually accompanied by a conversion of β to α chains. At a much earlier stage, however, the collagen exhibited a decreased reactivity with specific antibody and a decrease in its ability to form a water gel after prolonged dialysis against deionized water. Microbiological examination of solutions in which such changes occurred revealed the presence of molds.

The desired reduction in time of exposure to acid solutions was accomplished in two ways. Pressure filtration greatly accelerated the process of clarifying the citrate extract, a process previously accomplished with fritted-glass vacuum filtrations, and the use of solid KCl and Na_2HPO_4 in the same step eliminated a prolonged dialysis of the citrate-collagen solutions versus 1% NaCl to obtain fraction 2A.

For the purpose of testing the efficacy of the various precipitants in separating tropocollagen from other proteins in solution, samples of bovine serum albumin and bovine glycoprotein were iodinated with ^{131}I. Mixtures containing 0.1% of one of the iodinated proteins and 0.2% tropocollagen in 0.05% acetic acid were dialyzed against (a) 15% KCl, (b) 1% NaCl, and (c) 0.02 M K_2HPO_4. After centrifugation the supernatant solutions and the precipitates were separated. The latter were redissolved by dialysis against 0.05% acetic acid, and samples of the supernatant and the redissolved precipitates were assayed for ^{131}I. The results are given in Table I. Since under the dialysis conditions the albumin and glycoprotein are soluble, the contamination of the tropocollagen in the precipitate with ^{131}I indicates coprecipitation of the added protein. These results suggested that 1% NaCl is the most selective precipitant. After enzyme treatment, however, the modified tropocollagen no longer is precipitated by 1% NaCl and two to three successive precipitations with 15% KCl were used.

In the course of the isolation of the acid-soluble tropocollagen (fraction 2A) which was the main subject of subsequent study, other fractions (e.g., salt-soluble fraction 1) were also obtained and their properties are briefly described below.

Fraction 2A represented the bulk of the soluble collagen obtained. It was further purified by dissolution in 0.05% acetic acid, ultracentrifugation (<0.25% collagen solution at *ca.* 60,000 *g* for 30 minutes), and precipitation by dialysis against a large volume of cold 1% NaCl. This process was repeated two or three times. Complete recovery of the tropocollagen after NaCl dialysis was effected by allowing the flocculating suspension to warm to approximately 20°.

Analytical Correlates of Purification. The amount of tropocollagen in the skin extracts was measured by the difference (Δ) in specific optical rotation before and after thermal denaturation. During purification, the tropocollagen-nitrogen ratio was found to rise quickly (to a value $\Delta/N = -4800°/g$ nitrogen), but this measure gave a poor indication of purification during the final stages. Similarly, measurements of nitrogen content relative to the refractive-index increment of the solution (over its dialysate) quickly reached a plateau value. This measure would detect the elimination of nondialyzable polysaccharides and other low-nitrogen contaminants.

The tyrosine content decreased from 0.8% in the first citrate extracts to between 0.2 and 0.4% in the purified product, but the difficulty of measuring these small quantities made the tyrosine tests of limited use. The apparent hydroxyproline percentage, measured colorimetrically, increased from about 11% in the citrate extract to approximately 14% in the purified product; this, therefore, was one of the more useful criteria of purification.

Table II shows the amino acid composition of two different preparations of calfskin tropocollagen (fraction 2A) and of a sample from one of these preparations after pepsin treatment. These analyses were made on 20-, 40-, 70-, and 140-hour hydrolysates; the values

TABLE II: Amino Acid Composition of Tropocollagen and of Pepsin-treated Tropocollagen,[a] and of Pepsin-released Telopeptides.[b]

Amino Acid	Tropocollagen Fraction 2A		Pepsin Treated	Telopeptides	
	J4	J6[c]	J4 TC	Expt A	Expt B
Hydroxyproline	98.0	92.0	95.0	0	0
Aspartic acid	44.7	45.1	44.5	2.6	2.9
Threonine[d]	17.0	18.2	16.9	0	0
Serine[d]	33.6	36.8	35.1	1.5	1.5
Glutamic acid	72.2	71.7	70.0	4.0	4.7
Proline	123.0	120.0	125.0	2.7	2.3
Glycine	330.0	333.0	327.0	4.0	4.8
Alanine	111.0	115.0	116.0	0.8	1.2
Valine	22.2	21.6	22.2	0	0
Methionine + methionine sulfone	6.0	5.0	7.0	0	0
Isoleucine	11.3	11.2	11.5	0	0
Leucine	27.4	25.5	26.5	3.9	3.8
Tyrosine	3.5	3.8	1.3	3.6	4.4
Phenylalanine	12.8	13.4	11.6	3.7	3.3
Hydroxylysine	7.5	7.4	9.0		
Lysine	26.0	25.5	26.1	0	0.3
Histidine	4.4	4.3	5.9	0.5	0.6
Arginine	50.2	50.6	49.8	1.2	1.2
	1000.8	1000.1	1000.4		
Ammonia	30.0	30.0	30.0		

[a] Expressed as moles/1000 residues recovered from the column. [b] Expressed as mole residues recovered from column per mole of enzyme-treated tropocollagen. Molecular weight of tropocollagen, assumed 300,000. [c] Analysis of J6 (2A) gave hexose 0.4% (±0.05%) and hexosamine 0.16% (±0.01%). [d] Corrected for losses on hydrolysis (3% serine, 1% threonine per 20 hours).

were appropriately extrapolated to correct for destruction of serine and threonine and the slow hydrolysis of valyl and isoleucyl peptides.

Similarly complete analyses of the other fractions of tropocollagen have not been completed, but ultracentrifugal analyses of the fractions were made and the viscosities were measured. The characteristics of these preparations are summarized in Table III. Fraction 3 appeared to contain denatured tropocollagen or noncollagenous material.

Free-flowing and moving-boundary electrophoretic analysis showed only one component to be present in each of the calfskin tropocollagen preparations. No changes were detected in the properties of the tropocollagen after preparative electrophoresis.

The nitrogen content of calfskin tropocollagen fraction 2A, calculated from the composition (including hexoses), was 17.6%. On the assumption that there are no other undetected constituents of tropocollagen, this value was used in conjunction with Kjeldahl nitrogen determinations to calculate tropocollagen concentration in solution. The direct determination of the nitrogen content of tropocollagen was not attempted since the errors inherent in the nitrogen analysis (±1%) and the difficulties of adequately drying a protein preclude a more accurate measurement.

Effect of Conditions of Pepsin Treatment. The quantity of dialyzable peptides released by peptic treatment of native tropocollagen was studied as a function of time and of the enzyme-substrate ratio. These analyses were made by the Folin-Lowry (Lowry *et al.*, 1951) method. At all enzyme-substrate ratios down to 1:100 the action was essentially complete in 24 hours at 20°. At 1:1000 the reaction was 80% complete in that time. At a ratio 1:100 no additional peptide material was released between 24 hours and the termination of these experiments at 96 hours. The pepsin after 96 hours in 0.05% acetic acid was assayed and the activity was found unchanged.

Telopeptides Liberated from Tropocollagen by Pepsin. From the dialysate of pepsin-treated tropocollagen a solution of amino acids and peptide fragments corresponding in weight to approximately 1% of the tropocollagen was obtained. The amino acid content of the telopeptide material was significantly different from that of the parent tropocollagen. No hydroxyproline was detectable and the tyrosine percentage was many times higher than it was in the tropocollagen. The compositions of the telopeptide preparations obtained at *p*H 3.5

TABLE III: Comparison of Soluble Collagen Fractions Obtained during the Preparation of J6 Calfskin Collagen.

Fraction	Yield (g dry wt)	Composition (%) (α:β:γ)	$[\eta]_{20°}$[a]	$[\alpha]_{25°}^{365m\mu}$ (deg)	$[\alpha]_{45°}^{365m\mu}$ (deg)
1	0.1	53:33:14	12.0	−1320	−460
2A	17.7	35:62:3	13.8	−1305	−468
2B	7.0	44:50:6	14.6	−1310	−470
3	0.25	78[b]:22	5.1	−880	−465

[a] Sodium citrate (0.15 M; *p*H 3.7). Measurements in a simple Ubbelohde viscometer without corrections for shear dependence of the viscosity. [b] $\alpha + \beta$ + lower molecular weight components.

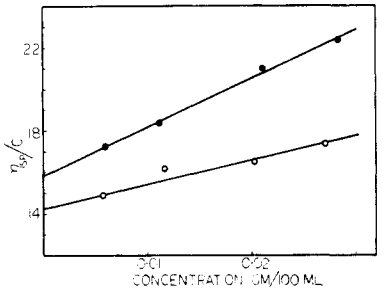

FIGURE 2: Viscosity (extrapolated to zero shear gradient) of calfskin tropocollagen before (●) and after (○) pepsin treatment. Three such experiments gave values of 13.6, 14.1, and 14.2 dl/g for the intrinsic viscosity of pepsin-treated tropocollagen. Two control tropocollagen preparations had values of 13.6 and 15.8, and both showed a higher concentration dependence.

are shown in Table II (see Rubin *et al.*, 1963, for a comparable experiment at *p*H 2.0). Similar amino acid patterns were obtained in ten analyses of dialysates from four different preparations.

Initial experiments were conducted at *p*H 2.0 in acetic-HCl, but at this *p*H pepsin is autolyzed; control experiments were necessary, therefore, to subtract the contribution of pepsin fragments from the collagenous peptidic material. Under the final conditions adopted, i.e., 0.05% acetic acid, *p*H 3.5, there was no evidence (from chromatograms of dialysates from pepsin incubated alone) that pepsin was autolyzed. Despite the large contribution at *p*H 2.0 of pepsin autolysis products, when the necessary corrections were made the amino acid composition of the dialyzable constituents released by pepsin from the collagen was consistent with that found in experiments conducted at *p*H 3.5. This consistency is in itself a reflection of the purity of the initial tropocollagen.

The material removed from the tropocollagen by pepsin treatment was dialyzed away from the residual tropocollagen, concentrated, and studied by chromatography. Ninhydrin spray revealed the presence of several compounds and staining by amido black showed a smear of at least four peptides which moved slowly on the paper. Adequate resolution of these peptides on paper or on cellulose acetate, by chromatography or by electrophoresis, was not achieved; their number cannot yet be defined. Analysis of the telopeptides by column chromatography has been performed and will be reported separately. The same distribution of peptidic material on paper chromatograms was observed in separate analyses of five preparations of the calfskin collagen. The control chromatograms of the material in the dialysates from the pepsin alone and from collagen untreated with pepsin showed no peptidic material moving from the origin.

Properties of Pepsin-treated Tropocollagen. The tropocollagen remaining after pepsin treatment showed altered properties. As shown in Figure 2, the intrinsic viscosity of the tropocollagen was lower after pepsin treatment, and the concentration dependence of the viscosity was modified. The ability of enzyme-treated collagen to give rise to a water gel on prolonged dialysis against deionized water was also severely inhibited, as we have previously reported (Rubin *et al.*, 1963). A more complete report on the influence of protease treatment on the physical properties of tropocollagen will be made subsequently (M. P. Drake, P. F. Davison, S. Bump, A. L. Rubin, and F. O. Schmitt, paper in preparation).

When purified preparations of tropocollagen are precipitated from acidic solutions by addition of ATP, they typically form a fibrous precipitate containing ribbonlike linear aggregates of the "segment-long-spacing" pattern. After protease treatment, with or without the removal of the residual protease, the form of the precipitate was characteristically flocculent, and electron-microscopic examination revealed almost entirely monomeric segments. This observation, previously noted by Hodge *et al.* (1960), is perhaps the most direct demonstration that pepsin modifies the intrinsic end-to-end interaction of the tropocollagen molecules. No evidence for the removal of any part of the tropocollagen molecule was discernible from electron-microscopic examination of the segment-long-spacing segments.

Most of these studies were made at enzyme-substrate

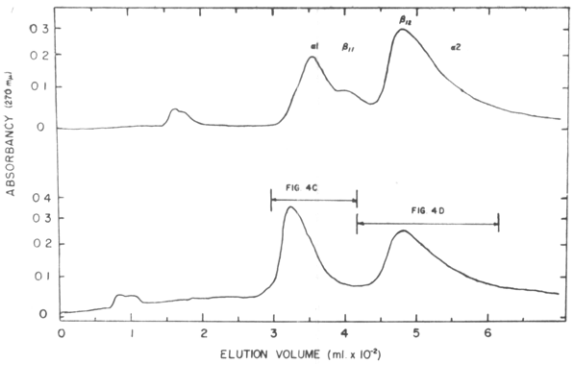

FIGURE 3: Elution diagrams (traced from the originals) of denatured tropocollagen on CM-cellulose. The 2.2 × 25–cm column was maintained at 40° and eluted with acetate buffer, *p*H 4.5, varying in concentration linearly from 0.08 to 0.25 M (with 350 ml of each buffer). Fractions of 10 ml were collected at a rate of 4 ml/min. The upper diagram was 22 mg of normal J4 tropocollagen, and the lower diagram was 18 mg of pepsin-treated J4 tropocollagen. The fractions were pooled as indicated by the arrows and their sedimentation patterns are shown in Figure 4.

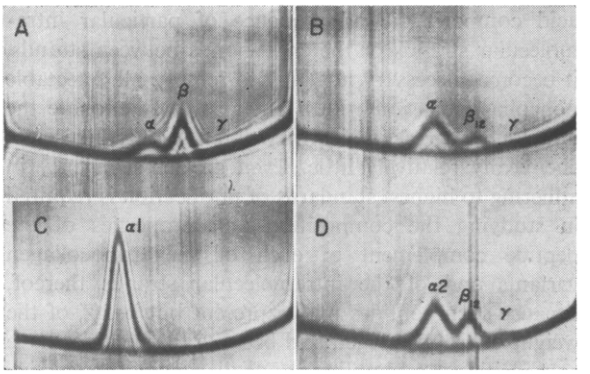

FIGURE 4: Sedimentation patterns (from left to right) of denatured tropocollagen run at 40°, 56,100 rpm, for 120 minutes; bar angle 60°. (A) Normal J4, concn 0.33% (α-β-γ ratio is 35:62:3); (B) pepsin-treated J4, concn 0.32% (α-β-γ, 74:23:3); (C) pooled first peak from CM-cellulose "hot column" of pepsin-treated J4, concn 0.52%; (D) pooled second peak from CM-cellulose "hot column" of pepsin-treated J4, concn 0.37%.

weight ratios of 1:25 and 1:100 (1:2 and 1:8 mole ratios, respectively). Since it might be postulated that interference in the aggregative properties of tropocollagen could result from specific or nonspecific combination with enzyme, these same digestions were performed at an enzyme-substrate weight ratio of 1:1000 (mole ratios of 1:80). The potentiality of tropocollagen to form a water gel was thereby abolished, and the usual complement of telopeptides was recovered after dialysis. Moreover, the pepsin was removed by electrophoresis (as described in the experimental section), and the properties of the tropocollagen were not changed. Finally, the addition of pepsin to intact tropocollagen under conditions in which the proteolytic activity is nearly zero (e.g., at 3°) did not appreciably influence the interaction properties since a gel was formed, as usual, upon water dialysis.

The ultracentrifuge diagram of denatured tropocollagen showed that a major fraction of the β component was converted into α chains after enzyme treatment. Similar experiments on samples of tropocollagen which had been enriched in the γ-component by denaturation, renaturation, and precipitation (to be published) showed that less than 10% of the γ was converted into α and β components. Experiments with hot-column chromatography (Piez *et al.*, 1961) on denatured protease-treated collagen showed that the $\beta_{1,1}$ fraction was quantitatively converted into α1 chains, and approximately half of the $\beta_{1,2}$ component is similarly degraded into α1 and α2 chains (Figures 3 and 4).

Other experiments have shown that changes in the aggregative properties of collagen similar to those effected by pepsin can be brought about by treatment of collagen with trypsin, chymotrypsin, elastase, and pronase. However the composition of the telopeptide material in these cases differs. These studies will be reported separately.

Discussion

The proteins belonging to the collagen class are widely distributed in animal tissues. They arose early in evolution, possibly even in the protozoa (Astbury, 1940, 1961). Distinguishing this protein from other classes of proteins is the three-strand helix structure and the high glycine and proline-hydroxyproline content. These properties are useful as criteria of purity in following the extraction of tropocollagen from tissues and its subsequent purification. However, unlike proteins such as insulin or hemoglobin, tropocollagen is not a clearly definable molecular entity but rather a family of closely related variants of a common basic structure. Though differences in chemical composition from one variant to another may be small, the differences in biochemical and physiological reactivity may be great. A similar situation in the case of variants of the fibrinogen-fibrin family has plagued investigators of blood clotting for almost a century. The systematic investigation of the tropocollagen variants is therefore not merely of academic interest, but is of practical biomedical significance.

The standards of preparation and purification of tropocollagen vary with the goal of the investigation. Thus it is possible to study the concentrations and intramolecular transitions of variants such as $\alpha\alpha\alpha$-, $\alpha\beta$-, and γ-tropocollagen in preparations containing appreciable amounts of noncollagenous impurities, comparable to the limits of sensitivity and reproducibility of the analytical tests for demonstrating the impurities. If, however, the goal is to investigate the amino

acid composition and sequence of particular intramolecular strands or the cross-bonds between strands, it becomes necessary not only to remove all detectable noncollagenous impurities, but also to fractionate the individual variants which may differ among themselves chemically relatively little. Even greater stringency in adhering to highest standards of purification is required in studying the composition and properties of the peptide complement of each of the tropocollagen variants and of the intramolecular strands thereof, because such peptides may represent but 1–5% of the weight of tropocollagen and because the peptides have compositions resembling noncollagenous connective tissue proteins more than they do tropocollagen; they may lack hydroxyproline. Unfortunately none of the analytical procedures employed in this study is capable of demonstrating impurities at a level of 1–2%. The reproducibility of the telopeptide analyses for different preparations must presently stand as the sole criterion of the purity of the tropocollagen. But because it is possible that the nonhelix peptides play a significant, possibly crucial, role in sequential alterations in tropocollagen during its "life history" from the biosynthesis of the individual α strands in the cell to the formation of mature, highly cross-linked fibers (and in the homeostatic regulation of collagen deposition in normal and pathological conditions), accelerated progress in the characterization of the peptides is urgent.

It would be helpful in facilitating such progress if agreement among experts could be reached concerning standards of extraction technique and of purity of the product. Publication by each investigator of analytical data characterizing the starting material is necessary if widespread disagreements in experimental results between scientists of unquestioned ability is to be avoided.

Customarily, extraction of tropocollagen with relatively mild reagents is pursued until the composition of the product is altered little by additional procedure or until the tropocollagen approaches a predetermined standard. Even so there may be disagreement about such elementary questions as to whether tyrosine is part of the tropocollagen molecule, as maintained in this laboratory, or arises from impurities, as claimed by Kühn and Zimmer (1961).

Functional as well as chemical analytical tests are also necessary as indices of the ability of the extracted and purified tropocollagen to form normal, native-type collagen fibrils in appropriate chemical and physical environments. In earlier phases of the investigations in this laboratory, substantial reliance was placed on a test designed to indicate the ability of tropocollagen "monomers" to aggregate linearly to form protofibrils, postulated by Hodge and Schmitt (1960) as occurring normally by specific interaction of peptides protruding from each tropocollagen end. The test used was the ability of tropocollagen solutions to undergo a great increase in relative viscosity when dialyzed against deionized water. Stiff gels may result depending on circumstances of the experiment.

This viscosity rise could be reproducibly reduced, or completely prevented by preliminary treatment with pepsin (Hodge *et al.*, 1960; Rubin *et al.*, 1963). There could be little doubt that the enzyme greatly reduced intermolecular interaction when the results illustrated by Rubin *et al.* (1963) were obtained. These results occurred only under carefully controlled conditions, however, and small variations in the *p*H of the water (CO_2 content) had a pronounced effect on the end viscosity, while traces of EDTA abolished the viscosity increase completely. Moreover, this viscosity behavior is difficult to interpret in molecular terms where charged macromolecules may interact specifically and electrostatically in solutions of low ionic strength. On the other hand, the test relied on by some investigators (e.g., Kühn *et al.*, 1961), namely, the formation of a gel of native-type collagen fibrils upon heating salt solutions of tropocollagen to *ca.* 35°, is also unsatisfactory as a criterion of normal fibrillogenesis even when reversibility after cooling, following varying periods of standing at 35°, is also tested (Gross and Kirk, 1958). Fibrils in such thermal gels, like those in tropocollagen from lathyritic animals, may manifest a quarter-stagger array, with characteristic intraperiod bands, but may fail to show other properties such as high tensile strength. Electrostatic bonding laterally between tropocollagen neighbors is doubtless an important factor in favoring the quarter-stagger configuration, but specific interaction between peptides may provide additional strong stabilizing forces favoring the native-type configuration. Obviously a reliable and easily applied physical as well as structural test, possibly involving stress-strain relations, is needed in assessing the integrity of extrahelix chemical groups during purification and fractionation of tropocollagen and in investigating the effect of pathological or experimentally imposed alterations.

Turning to the second aspect of this paper, we propose preliminary interpretation of the results obtained from the pepsin treatment of tropocollagen. Full interpretation of the effect of scission from tropocollagen of extrahelix peptides by proteases must await completion of our experiments with other noncollagenase proteases. For example, since the amount of peptide material liberated, without alteration of the ability of the tropocollagen sample to form seemingly normal segment-long spacing, may be as high as 4–5%, as compared with 1% for pepsin action, it is obvious that peptides may be much more widely distributed about the tropocollagen molecule than is suggested from the original hypothesis of elongate end-peptides (Hodge and Schmitt, 1958; Schmitt and Hodge, 1960) or from the definitive analytical data herein reported for pepsin action. Indeed it is not possible to exclude the possibility that many short peptides protrude from the main strands at discontinuities such as have been implied by the various subunit hypotheses (e.g., Gallop, 1964; Petruska and Hodge, 1964).

After pepsin treatment the amino acid analysis of the hydrolyzed telopeptides corresponds roughly to 1% of the total tropocollagen in our acidic extracts (i.e., approximately 30 residues/mole). Hörmann and Hafter (1964), whose work largely confirms that of Rubin *et al.*

(1963), obtained a peptide nitrogen of 1.9% of the tropocollagen nitrogen. T. Nishihara and T. Miyata (private communication, 1962) and Hörmann and Hafter (1964) both found small amounts of hydroxyproline in hydrolysates of telopeptides. This would of course result if, at any stage of the experiment, a portion of the helix had become denatured, or if trace amounts of tropocollagen were allowed to contaminate the outside of the dialysis bags. The latter artifact may be eliminated simply by redialyzing the concentrate of the original dialysate containing the telopeptides. In our own experiments, though there were minor differences in the amino acid concentrations in the hydrolysates of dialysates after pepsin action, hydroxyproline was positively identified in none of them. This supports the view that the peptide material originated outside the helix portion of the tropocollagen.

It is impossible to say whether the 30–35 amino acid residues (see Table II) comprising the pepsin-liberated material preexisted as one strand or were part of a larger number of appendages which underwent peptic scission. There is evidence for some four peptides in the electrophoresis pattern, but many others have been detected by column chromatography. Their isolation and sequence analysis is now in progress. The presence of tyrosine in the dialysate hydrolysate, confirmed by Hörmann and Hafter (1964) who believed it to be a constituent of at least five of the twelve chromatograph spots, is of interest in view of the fact that the serological reaction of purified tropocollagen is reduced by pepsin treatment and that tyrosine frequently is associated with the antigenicity in proteins (see Arnon and Sela, 1960). The significant relationship between peptides, serological properties, and certain aspects of autoimmune and so-called "collagen diseases," suggested by Rubin *et al.* (1963), is being investigated and will be reported in due course.

The question whether the peptide or peptides acted on by pepsin are bonded covalently to the tropocollagen or are more loosely associated, as would be the case if the material were from an ubiquitous noncollagenous "impurity" (Kühn, 1962a), cannot be answered definitively though the evidence favoring covalent bonding seems convincing. The peptide attachment survives extensive preparative extraction and dialyses and is not separated by free-flow electrophoresis at several *p*H values. It has been reported that no free amino groups at the ends of the three polypeptide strands are demonstrable in purified tropocollagen, but after the action of pepsin, Hörmann and Hafter (1964) found evidence for end amino groups in concentration possibly explicable by scission of a peptide bond on one of the three strands. From our evidence this bond would have to be external to the helix portion, but might be part of a grouping that interbonds two α chains since pepsin action converts some of the β chains to α chains. Though the specificity of the tropocollagen-antitropocollagen reaction in the serological experiments of Schmitt *et al.* (1964) resides in the telopeptides, complement fixation is lost if the helix structure is disturbed, as by denaturation. Therefore the total sites recognized by antibody include both the telopeptides and the triple helix. Moreover, some complement fixation reaction is recovered on renaturation (to be published). It seems doubtful that this would occur if the peptide and the helix portions were not part of a single covalently bonded molecule.

Recent electron-microscopic studies suggest that, in the ordered aggregation of tropocollagen to form native-type collagen, the tropocollagen macromolecules do not form end-to-end unions, as had been supposed in the original protofibril hypothesis and as was suggested definitively by Schmitt and Hodge (1960) in proposing their end-peptide-interaction hypothesis; rather there is overlap of axial neighbors at ends (Hodge and Petruska, 1963; Kühn, 1962b; Olsen, 1963). However this evidence does not disprove the presence of peptides protruding from the ends or sides of the helix portion of tropocollagen, nor does it bear directly on the hypothesis that protruding peptides may be physiologically significant in fibrillogenesis through the formation of intermolecular cross-links, particularly if the peptide functions are relatively short and interact at multiple points along the lateral interfaces between tropocollagen macromolecules.

In sum, the evidence presented in this paper supports the position adopted previously in this laboratory that paucimolecular appendages protruding from tropocollagen proper, i.e., from the organized, triple-helix portion of the molecule responsible for the properties characteristic of proteins of the collagen class, play an important, possibly determinative role in the molecular biology of normal and pathological collagenous systems.

Acknowledgments

The invaluable assistance of Mr. J. W. Jacques, Miss A. Holzer, Mrs. H. Lengyel, and Mrs. E. Myers is gratefully acknowledged.

References

Anson, M. L. (1939), *J. Gen. Physiol. 22*, 79.
Arnon, R., and Sela, M. (1960), *Biochem. J. 75*, 103.
Astbury, W. T. (1940), *J. Intern. Soc. Leather Trades' Chemists 24*, 69.
Astbury, W. T. (1961), *J. Soc. Leather Trades' Chemists 45*, 186.
Bernhart, F. W. (1938), *J. Biol. Chem. 123*, x.
Boas, N. F. (1953), *J. Biol. Chem. 204*, 553.
Gallop, P. M. (1964), *Biophys. J. 4*, 79.
Gilmore, R. C., Robbins, M. C., and Reid, A. F. (1954), *Nucleonics 12*, 65.
Gross, J., and Kirk, D. (1958), *J. Biol. Chem. 233*, 355.
Hannig, K. (1961), *Z. Anal. Chem. 181*, 244.
Hodge, A. J., Highberger, J. H., Deffner, G. G. J., and Schmitt, F. O. (1960), *Proc. Natl. Acad. Sci. U.S. 46*, 197.
Hodge, A. J., and Petruska, J. A. (1963), Aspects of Protein Structure, Ramachandran, G. N., ed., London and New York, Academic, p. 289.

Hodge, A. J., and Schmitt, F. O. (1958), *Proc. Natl. Acad. Sci. U.S. 44*, 418.
Hodge, A. J., and Schmitt, F. O. (1960), *Proc. Natl. Acad. Sci. U.S. 46*, 186.
Hörmann, H., and Hafter, R. (1964), *Leder 15*, 293.
Kohn, R. R., Bensusan, H. B., and Klein, L. (1964), *Science 145*, 186.
Kühn, K. (1962a), *Leder 13*, 73.
Kühn, K. (1962b), *Leder 13*, 86.
Kühn, K., Hannig, K., and Hörmann, H. (1961), *Leder 12*, 237.
Kühn, K., and Zimmer, E. (1961), *Z. Naturforsch. 16*, 648.
Lowry, O. H., Rosebrough, N. J., Farr, L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Markham, R. (1942), *Biochem. J. 36*, 790.
Martin, C. J., and Axelrod, A. E. (1953), *Proc. Soc. Exptl. Biol. Med. 83*, 461.
Martland, M., and Robison, R. (1926), *Biochem. J. 20*, 847.
Olsen, B. R. (1963), *Z. Zellforsch. mikroskap. Anat. 59*, 199.
Petruska, J. A., and Hodge, A. J. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 871.
Piez, K. A., Lewis, M. S., Martin, G. R., and Gross, J. (1961), *Biochim. Biophys. Acta 53*, 596.
Rubin, A. L., Pfahl, D., Speakman, P. T., Davison, P. F., and Schmitt, F. O. (1963), *Science 139*, 37.
Sanger, F., and Thompson, E. O. P. (1963), *Biochim. Biophys. Acta 71*, 468.
Schmitt, F. O. (1960), *Bull. N.Y. Acad. Med. 36*, 725.
Schmitt, F. O. (1963), *Leder 14*, 21.
Schmitt, F. O. (1964), *Federation Proc. 23*, 618.
Schmitt, F. O., and Hodge, A. J. (1960), *Leder 11*, 74.
Schmitt, F. O., Levine, L., Drake, M. P., Rubin, A. L., Pfahl, D., and Davison, P. F. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 493.
Seifter, S., Seymour, D., Novic, B., and Muntwyler, E. (1950), *Arch. Biochem. Biophys. 25*, 191.

Bradykinin: Configurations of the Arginine Moieties and Biological Activity*

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ABSTRACT: Three new analogs of bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) were prepared which contained arginine in the D configuration: 1-D-arginine, 1,9-bis-D-arginine, and 9-D-arginine bradykinin. The last analog was found to be contaminated with about 5% of bradykinin, but treatment with carboxypeptidase B eliminated this impurity. Bioassay of the three analogs in the dog hindquarters has shown them to have negligible vasodilatory activity. These results indicate that the two terminal arginines of bradykinin must be of the L configuration in order for the molecule to exhibit its characteristic biological effects. The analogs did not show any inhibitory effects to bradykinin.

The importance of the bradykinin C-terminal arginine (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) for biological activity has previously been demonstrated since its removal results in significant loss of activity (Elliott *et al.*, 1960; Nicolaides *et al.*, 1963a). It has also been shown that synthetic bradykinin is destroyed in blood by a carboxypeptidase-type enzyme that cleaves the C-terminal arginine (Erdös, 1961). This enzymatic inactivation process is very rapid and probably contributes heavily to the extremely brief biological half-life of bradykinin which has been found to be less than 0.5 minute in man (Saameli and Eskes, 1962) and 0.3 minute in the dog (D. A. McCarthy, D. E. Potter, and E. D. Nicolaides, submitted for publication).

In an effort to circumvent this rapid carboxypeptidase inactivation it appeared reasonable that introduction of an unnatural peptide bond at the point of attack would increase the biological half-life of the kinin. A somewhat similar approach has recently been described (Stewart and Woolley, 1964) for bradykinin in which the 8-phenylalanine was replaced by leucine. Although one would expect this type of peptide bond to be resistant to a chymotrypsinlike attack it would not be expected to provide protection from a carboxypeptidase cleavage.

Experimental

A half-life study of the 9-D-arginine analog would indicate the extent to which an aminopeptidase or other enzymatic attack was contributing to the inactiva-

* From the Parke, Davis and Company Research Laboratories, Ann Arbor, Mich. *Received September 22, 1964.*