exert an effect on peroxidase itself, as the non-enzymic reactions are not at all retarded at the C_{25} values given. We suggest that the seeming paradox may be resolved if the ethers exert their antioxidant effect on steps of intermediates unique to the enzyme-catalyzed oxidation.

The enzymic oxidation of pyrogallol in acidic solutions yields the trihydroxybenztropolone purpurogallin, whereas autoxidation under otherwise similar conditions, forms instead a variety of quinonoid products, principally of polymeric character.[8] The enzyme, then, restricts the alternative route(s) leading to the formation of quinones and polyquinones. We have observed that the polymerization of aqueous-*p*-benzoquinone in air is not affected by the presence of the ethers at concentrations up to 0.01 *M*. Thus, a model process for the formation of highly colored products during autoxidation is shown to be ether-insensitive. Although the oxidation of iodide to iodine by H_2O_2 is an ionic reaction which proceeds through formation of HOI, radical pathways have been implicated in its photooxidation.[9] Thus the oxidation of each of these substrates may proceed along at least two pathways, one of which may be favored in the presence of peroxidase and particularly ether-sensitive.

Although questions pertaining to mechanism remain to be answered, these data show clearly that simple ethers can act as oxidation inhibitors in a model biochemical system. The effective concentrations are beyond the hormonal range but may reflect a physiologically important property of the aryloxy group. Fungistatic activity and similar biological effects which require relatively high ether concentration may, on the other hand, be more directly explained by the foregoing observations.

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Experimental

Iodine formation (as I_3) was followed photometrically at 360mμ (Bausch and Lomb spectronic 20 Spectrophotometer); purpurogallin formation was followed at 425mμ. The C_{25} and R_{25} values were based upon triplicate measurement at 25° made during the initial, linear phase of the respective reactions—20 min. with iodide and 3 min. with pyrogallol.

The iodide system contained $5 \times 10^{-3}M$, KI $5 \times 10^{-4}M$ H_2O_2, and $2 \times 10^{-9}M$ horseradish peroxidase (Nutritional Biochemicals Corp.) in *M*/6 phosphate buffer, *p*H 5.6. The pyrogallol system contained $5 \times 10^{-3}M$ pyrogallol, $10^{-3}M$ H_2O_2 and $2 \times 10^{-8}M$ peroxidase in *M*/30 phosphate buffer, *p*H 5.0.

The polymerization of *p*-benzoquinone to red-brown products in air was followed at 500 mμ.[8] Reactions were run in the dark for 20 hr. at 25°. Freshly prepared aqueous solutions containing 0.005–0.05 *M* quinone and *M*/15 phosphate buffer, *p*H 5.0, were used.

The only problems of purity were encountered with phenyl ether, which was redistilled, and *p*-benzoquinone, which was recrystallized from ethanol. No phenols were detected in the phenyl ether in tests with $FeCl_3$ and NaOH. Fresh diethyl ether which gave a negative KI test for peroxides was used for each experiment.

Acknowledgments.—The authors wish to thank their colleagues Dr. V. Schomaker, who originally suggested that we test aromatic ethers, and Dr. R. L. Hinman, who read the manuscript critically and suggested the inclusion of tyrosine.

[CONTRIBUTION FROM THE PHYSICAL BIOCHEMISTRY DIVISION, NAVAL MEDICAL RESEARCH INSTITUTE AND THE DEPARTMENT OF BIOCHEMISTRY, ALBERT EINSTEIN COLLEGE OF MEDICINE, YESHIVA UNIVERSITY]

An Enzymatic Examination of the Structure of the Collagen Macromolecule[1]

BY PETER H. VON HIPPEL,[2] PAUL M. GALLOP, SAM SEIFTER AND ROBERT S. CUNNINGHAM

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The collagenase-catalyzed degradation of soluble ichthyocol has been examined in order to obtain further insight into the structure and configuration of the collagen macromolecule. The kinetics of proteolysis were followed directly by *p*H-stat and colorimetric ninhydrin methods, and it is shown that below 27° (T_c—the temperature at which the collagen → gelatin transition takes place in ichthyocol solutions) the over-all kinetics can be reduced to the sum of two concurrent reactions, both apparently first order in substrate concentration but differing markedly in rate. Since conversion to gelatin reduces the kinetics of proteolysis to a single, apparent first order reaction with a much smaller apparent energy of activation,[25] the complex kinetics observed at temperatures below T_c have been interpreted in terms of local differences in polypeptide chain configuration in the vicinity of the susceptible peptide bonds. Following an examination of the molecular properties of undegraded ichthyocol in neutral salt solution (0.5 *M* $CaCl_2$), the changes produced in the substrate as a consequence of proteolysis were monitored by various physico-chemical techniques. The specific viscosity of ichthyocol solutions falls rapidly (to less than 10% of its initial value) during collagenolysis, also following apparent first order kinetics. Parallel light-scattering experiments revealed that this fall is accompanied by only a slight decrease in molecular weight, but by a marked change in over-all particle shape—the macromolecules becoming more flexible as the reaction proceeds. The non-dialyzable protein concentration and specific rotation of the ichthyocol solution also fall much more slowly than the specific viscosity, indicating that the particle remains relatively unchanged in terms of size and helical content during the early stages of proteolysis. These results are interpreted in terms of a rigid, multi-stranded, inter-chain hydrogen-bonded structure for the collagen macromolecule in solution; enzymatic cleavage of single strands leaves the particle relatively intact but brings about a partial structural collapse by introducing points of increased flexibility.

Introduction

Collagen, the major protein constituent of connective tissue, has been studied for many years. However most examinations have been confined to collagen in the solid state or to degradation products (collectively termed gelatin), chiefly because of the resistance of collagen to solubilization under mild conditions. In 1927, Nageotte reported the first successful preparation of soluble

(1) Presented in part at the 3rd Annual Meeting of The Biophysical Society, February 25, 1959, Pittsburgh, Pennsylvania. The opinions expressed in this article are those of the authors and do not necessarily reflect the opinions of the Navy Department or the naval service at large.

(2) Department of Biochemistry (Biophysics), Dartmouth Medical School, Hanover, New Hampshire.

collagen,[3] but physico-chemical studies were not reported until 1950 when Bresler, *et al.*,[4] presented results obtained with a soluble skin collagen, prepared by Orekhovich, *et al.*,[5] using a citrate extraction procedure. Subsequently, Gallop showed that a particularly pure soluble collagen, termed ichthyocol, could be isolated by subjecting the tunics of carp swim bladders to citrate extraction.[6] This collagen, dissolved in *p*H 3.7 citrate buffer, and the parent gelatin produced from it by mild heating, have since been the object of extensive physico-chemical examination by Gallop,[6,7] Cohen[8] and especially Boedtker and Doty.[9] From these studies it has been concluded that ichthyocol collagen exists in this solvent as rigid rod-like, three-stranded macromolecules (molecular weight approximately 4×10^5) and that these macromolecules dissociate on heating to form the single-chain, essentially randomly coiled molecules of parent gelatin.[10]

Recently, a very active collagenase has been prepared and purified from *Clostridium histolyticum*.[15,16] This enzyme seems to be highly specific for collagen and gelatin severing these proteins at amino acid sequences having the general formula -Pro.X.Gly.Pro.Y-, between X and Gly,[16-19] while attacking no other natural substrate so far tested. Since collagenase is activated by ionic calcium[15,20] and inactivated by low *p*H, acidic citrate buffer could not be used as a collagen solvent for studies with this enzyme. However this difficulty was overcome by the demonstration by several groups of workers that collagen can be solubilized by treatment with concentrated solutions of neutral salts[21-23]—0.5 *M* $CaCl_2$ being particularly suitable for enzymatic studies.

In this communication we consider in detail the degradation of soluble ichthyocol by purified collagenase, in order to obtain further insight into the structure of the collagen macromolecule. Two general approaches were used in this work: (1) the kinetics of proteolysis under various experimental conditions were analyzed and the results interpreted in terms of the enzyme as a "probe" of local polypeptide chain configuration (see also refs. 24, 25) and (2) the molecular changes which accompany collagenolytic attack were monitored and an attempt made to elucidate certain features of the intact macromolecule on the basis of these changes.

Materials and Methods

Collagen.—Ichthyocol was prepared from carp swim bladders by extraction with acidic citrate buffer, followed by "reconstitution" by dialysis.[6] The purified collagen was lyophilized and stored over anhydrous $CaCl_2$ at 5°. Stock solutions of ichthyocol were made by suspending the dried material in *p*H 7.0, 0.5 *M* $CaCl_2$–0.05 *M* Tris (or unbuffered 0.5 *M* $CaCl_2$, adjusted to *p*H 7.0) by brief homogenization in a Teflon-glass tissue homogenizer at cold room temperatures, followed by gentle stirring for 24 hr. at 5°. Undissolved residues were removed by centrifugation (3 hr. at 22,000*g* in the Spinco Model L Preparative Ultracentrifuge). The resulting clear stock solution, stored in polyethylene bottles at 5°, was stable for periods of several weeks.

Collagenase.—Bacterial collagenase was isolated from cultures of *Cl. histolyticum*, purified by methods described elsewhere[15,16] and stored as a lyophilized powder. A stock solution was made by dissolving this material in distilled water at a concentration of 2.5 mg./ml. One ml. portions of this stock solution were quick-frozen in small tubes, stored at −20° and diluted for use as needed. In the frozen state the enzyme is stable almost indefinitely and in solution the activity remains constant for well over a month. The collagenase activity of the stock solution was determined viscometrically, using soluble ichthyocol collagen as a substrate.[15] The stock solution used in these studies had an activity of 250 units per ml. (see ref. 15 for a discussion of collagenase activity units).

Protein Concentration.—Protein concentrations were determined using the Folin-biuret procedure of Lowry, *et al.*,[26] as modified by Gellert, *et al.*[27] Before measurement, ichthyocol collagen was converted to gelatin by heating to 60° for 10 minutes and then dialyzed against distilled water to remove ionic calcium (which interferes with the test by forming insoluble calcium hydroxide precipitates). The procedure was standardized against micro-Kjeldahl determinations of protein nitrogen on several preparations of ichthyocol; a protein nitrogen factor of 17.0% was used in the micro-Kjeldahl calculations. This Folin-biuret technique is accurate to ±2% at protein concentrations in excess of 0.1 mg./ml.

***p*H-stat Measurements.**—The collagenolytic cleavage of peptide bonds was followed by a manual "*p*H-stat" method, using a Beckman Model H *p*H-meter equipped with calomel and glass micro-electrodes. The *p*H was maintained constant with standardized KOH (approximately 0.01 *M*) delivered from a calibrated microsyringe, and CO_2 contamination was prevented by continuous passage of water-saturated, ammonia-free nitrogen gas over the solution. The entire reaction vessel was immersed in a thermostated bath (±0.05°) and stirred continuously. In a typical experiment, 20 ml. of 0.5 *M* $CaCl_2$ were placed in the reaction vessel, brought to temperature and "flushed" with nitrogen.

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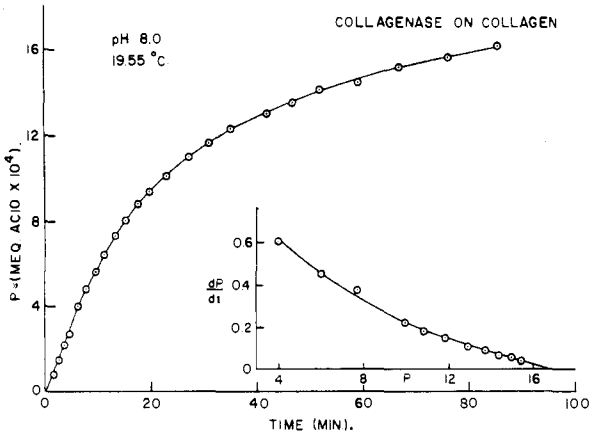

Fig. 1a.—Meq. acid (P) produced by the action of collagenase on ichthyocol, as a function of time. pH 8.00, 19.5°; collagen concentration, 0.16 mg./ml.; collagenase concentration, 0.45 units/ml. Insert: Rate of acid production (dP/dt) as a function of P. (Data from upper curve). $P_\infty = 17.05 \times 10^{-4}$ meq. acid.

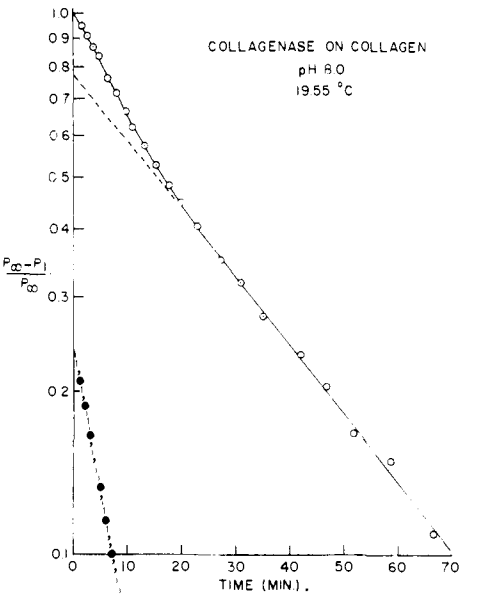

Fig. 1b.—Fraction of the total bonds cleaved (logarithmic scale) as function of time. Data from Fig. 1a.

Then 5 ml. of ichthyocol stock were added with a precooled pipette and after thermal equilibration the solution was adjusted to the proper pH. At zero-time collagenase was added rapidly using a micropipette or syringe, the pH was restored as quickly as possible, and maintained constant by appropriate additions of base thereafter. The amount of enzyme added in each experiment was generally chosen to bring the reaction to 90% of completion within about 90 minutes.

The differential titrations of collagenase-digested and undigested ichthyocol, as well as the titrations of the N-terminal glycine peptides, were performed using the TTT-1 Automatic Titrator and the SBR2/SBU1 Titrigraph supplied by Radiometer, Copenhagen, Denmark.

Colorimetric Ninhydrin Measurements.—Peptide bond cleavage was also monitored, in some cases, by taking samples of the digestion mixture at various times after adding enzyme and assaying for free α-amino groups by the ninhydrin method of Rosen.[28] Control experiments using N-terminal glycine peptides (obtained from Mann Research Laboratories) were made to ascertain the colorimetric equivalence of the α-amino groups of the free amino acids used as standards and α-amino groups located at the ends of polypeptide chains; such models were employed because the peptides resulting from the collagenolytic digestion of collagen all seem to carry N-terminal glycine.[17] In all cases the optical density of the peptide solutions was 90 to 100% of that obtained with an equivalent concentration of free glycine.

Viscosity.—All measurements of the specific viscosity of ichthyocol solutions during collagenolytic degradation, as well as some determinations of the intrinsic viscosity of ichthyocol, were carried out in commercial, size 100 Ostwald-Cannon-Fenske capillary viscometers, obtained from Fisher Scientific Company. These viscometers had an average out-flow time for water of approximately 70 seconds and an average shear gradient (β) of about 1400 sec.$^{-1}$ for water at 20°. The intrinsic viscosity of ichthyocol in solution was also determined using a low-shear viscometer (β = 185 sec.$^{-1}$ for water at 20°) constructed by Mrs. Ann Ginsburg of the National Institutes of Health, Bethesda, Md. Temperatures were regulated to ±0.05°. Protein solutions used were generally pre-centrifuged at 30,000g for about 2 hr. Only small volumes of enzyme solution (generally 0.5 ml. or less) were used in the experiments in which viscosity changes were utilized to follow the progress of proteolysis; these solutions were thermally pre-equilibrated and viscosity measurements were begun immediately after addition.

Light-Scattering.—Light-scattering measurements were carried out in a Brice-Phoenix Light-Scattering Photometer, using 436 mμ incident light and a beam collimated to a width of 4 mm. Relative scattering intensity measurements were made at angles ranging from 27° (sometimes 21°) to 135° relative to the undeviated beam, using cylindrical cells with planar entrance and exit windows. Square turbidity cells were employed when scattering measurements at 90° only were required. The absolute calibration of the instrument, details of cell calibration, reflectance corrections, etc., have been discussed elsewhere.[27] A specific refractive index increment (dn/dc) of 0.187 ml./g., measured by Boedtker and Doty[9] on ichthyocol collagen and gelatin in citrate buffer, was used in the calculations.

Solvents intended for use in light-scattering experiments were optically clarified by several passages through millipore filters. The clarification of ichthyocol solutions for light-scattering, as Boedtker and Doty have pointed out,[9] presents certain difficulties. In order to obtain reproducible results the following rather elaborate procedure was adopted. The protein solutions to be used (protein concentration <2 mg./ml.) were subjected to an initial 2-hr. ultracentrifugation (Spinco Model L) at 30,000g, the upper portion of each was carefully drawn off (siliconized pipette), diluted to the proper concentration with filtered buffer in a pre-rinsed Sorval centrifuge tube and recentrifuged for approximately 90 minutes in the Sorval centrifuge (SS-1) at 20,000g. Then, without removing the tube from the rotor, the upper portion of each solution was again carefully drawn into a large siliconized pipet, using a special siphoning device, and transferred to the scattering cell. All solutions were inspected visually in the beam for forward scattering from residual "dust"; if such scattering was observed, the clarification procedure was repeated. Enzyme solutions intended for use in light-scattering experiments were clarified by ~3 hr. of ultracentrifugation at 80,000g. Generally protein concentrations were redetermined on light-scattering solutions after optical clarification had been completed.

In the absence of a satisfactory thermostated light-scattering cell, the cells were maintained in appropriate water-baths and placed in the photometer only long enough to make the measurements. Because elevated temperatures both accelerate the rate of enzyme action and favor ichthyocol denaturation, the proteolysis experiments were carried out in a thermostated bath (±0.05°) at 8 to 15°, and both the bath and the photometer were placed in a 5° cold room. Thus temperature changes during measurement were minimized. The modifications found necessary to operate the Phoenix Light-Scattering Photometer under cold room conditions have been described elsewhere.[27]

Full Zimm plots,[29] based on measurements at several concentrations, were made on solutions of ichthyocol in 0.5 M $CaCl_2$ (see Fig. 4) and revealed that Kc/R_θ is essentially independent of concentration. Therefore, in subsequent experiments the data obtained during enzymatic degradation

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were plotted against $\sin^2(\theta/2)$ only, corresponding to the limiting zero-concentration envelope of a conventional Zimm plot.

Optical Rotation.—Optical rotation measurements were made in thermostated (±0.1°) one decimeter Rudolph #18 jacketed polarimeter cells at a wave length of 589 mμ, using the Keston polarimeter attachment to the Beckman DU spectrophotometer.

Results and Discussion

I. Kinetics of Proteolysis. *p*H-stat Measurements.—The proteolysis of collagen by collagenase was monitored by measuring the appearance of new α-amino groups by *p*H-stat and by colorimetric ninhydrin methods and both yielded essentially identical data. A typical *p*H-stat experiment is presented in Fig. 1a. The upper curve represents meq. of acid produced (P) as a function of time after adding collagenase. In the insert, the rate of proteolysis (dP/dt) is plotted against P_t, and the final points are extrapolated linearly to the abscissa to obtain P_∞, the meq. of acid produced when the reaction has gone to completion.[30] These data are plotted as a first order reaction in Fig. 1b. Along the ordinate, on a logarithmic scale, we plot $(P_\infty - P_t)/P_\infty$, the fraction of susceptible bonds remaining uncleaved at time t. Note that the experimental data, after an accelerated initial portion, follow apparent first order kinetics quite accurately over several half-lives. Linear extrapolation of the latter portion of the reaction back to the ordinate yields the upper, dashed, straight line. Subtraction of this line from the experimental curve results in the dashed difference curve plotted in the lower part of the graph. Note that the difference curve also follows apparent first order kinetics.

Similar runs were made at 2 to 3° intervals between 10 and 25°, and in all cases a similar analytic pattern was obtained. Each time the experimental data could be reduced to the sum of two apparent first order reaction classes, each linear (when plotted as a first order reaction) over two to three half-lives. Thus it would appear that the collagenase-susceptible peptide bonds of ichthyocol can be divided into two classes on the basis of reactivity with the enzyme—bonds of both classes being cleaved in apparent first order fashion but at markedly different rates. The fraction of the bonds split in each reaction was determined by linear extrapolation of the dotted lines to the ordinate, while apparent first order rate constants were derived from the slopes.[31] These data, for a series of experiments at different temperatures, are compiled in Table I.

In Fig. 2, the logarithms of the apparent first order rate constants (k) obtained from a series of such *p*H-stat runs are plotted against the reciprocal of the absolute temperature, in the form of an Arrhenius plot. The lines numbered (2) and (3) correspond to the data obtained on ichthyocol at

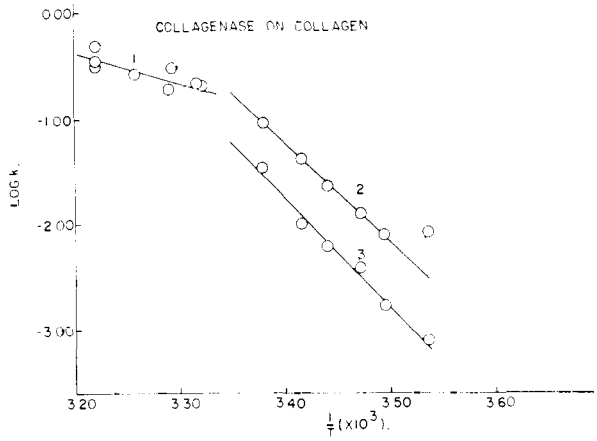

Fig. 2.—Arrhenius plot of the effect of collagenase on ichthyocol and on gelatin derived from it by mild heating (10 min. at 50°) *p*H-stat data. 1—Gelatin above T_c; 2—collagen below T_c, fast reaction; 3—collagen below T_c, slow reaction (see text).

temperatures below 27°. All runs were made at the same initial substrate concentration (0.16 mg./ml.) but for convenience progressively larger amounts of enzyme were used at the lower temperatures. Thus for comparative purposes, the rate constants plotted in Fig. 2 have been normalized to an enzyme-substrate ratio of unity.[25] The data

TABLE I

KINETIC DATA FROM *p*H-STAT RUNS, COLLAGENASE ON ICHTHYOCOL

Temp. (°C.)	k' (fast),[a] $\times 10^2$ min.$^{-1}$	k' (slow)[a] $\times 10^2$ min.$^{-1}$	% Bonds split Fast react.	% Bonds split Slow react.
22.7	9.2	3.4	11	90
19.6	4.3	1.0	23	77
17.5	2.4	0.64	17	83
14.9	1.3	.39	19	82
13.0	0.80	.22	16	85
9.7	0.84	.083	14	86

[a] Adjusted to an enzyme-substrate ratio of unity (see refs. 25, 32 and text).

obtained fall onto two straight lines—line (2) corresponding to the fast reaction and line (3) to the slow. The apparent energies of activation (ΔE_a^*) for each reaction may be derived from the slopes of these lines and are presented in Table II. By applying transition state theory[33,34] to these data, the apparent enthalpy ($\Delta H^\ddagger$), free energy ($\Delta F^\ddagger$) and entropy ($\Delta S^\ddagger$) of activation for each reaction may also be calculated. These quantities are also compiled in Table II.

The temperature (T_c) above which ichthyocol collagen undergoes conversion to single-chain, randomly coiled parent gelatin, is about 27°.[9,25,35] Kinetic data obtained on ichthyocol gelatin at temperatures above T_c in a previous study of the gelatin → collagen-fold transition[25] are included in Fig. 2 and Table II for comparative purposes.

(30) The use of such a linear extrapolation to obtain P_∞ implies that the reaction follows apparent first order kinetics, at least during the later stages (see ref. 25 and below).

(31) The apparent first order nature of the reactions observed in experiments of this sort, over a range of initial substrate concentrations,[32] has interesting implications for the mechanism of collagenase action and perhaps for proteolysis kinetics in general. A detailed discussion of the kinetics of proteolysis of collagen and gelatin by collagenase at various substrate and enzyme concentrations, *p*H values, etc., will be presented elsewhere.[32]

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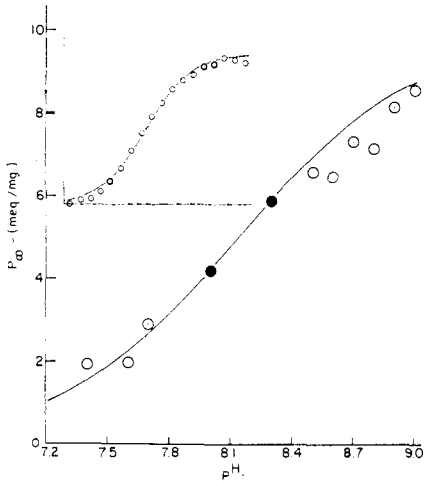

Fig. 3.—Meq. acid (P_∞) produced during entire reaction, versus pH. Collagenase on ichthyocol gelatin at 30°, 0.5 M $CaCl_2$. Curve based on solid points (●); pK'_{30} = 8.13, N = 8.8 μM α-amino groups. Insert: Differential titration, ichthyocol sample completely digested with collagenase minus undigested control, ~30°, 0.5 M $CaCl_2$. Experimental points, average of two runs; curve calculated for pK' = 8.1, N = 3.5 μM α-amino groups.

Note the sharp break in the Arrhenius plot at the conversion temperature, as well as the fact that above T_c all of the collagenase-susceptible peptide bonds appear to be split in a single apparent first order reaction, with $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (~15 kcal./mole and ~0 e.u., respectively) close to the values

TABLE II

THERMODYNAMIC DATA, COLLAGENASE ON ICHTHOCOL

Reaction[a]	ΔE_a* (kcal./mole)	$\Delta H^{\ddagger}$ (kcal./mole)	$\Delta F^{\ddagger}$ (kcal./mole)	$\Delta S^{\ddagger}$ (e.u.)
1	15	+14	+14	+1 ± 7
2	42	+41	+15	+90 ± 5
3	47	+46	+15	+110 ± 6

[a] See Fig. 2 and text for identity of reactions and temperature ranges.

usually associated with the proteolysis of denatured proteins and synthetic substrates.[36] The same quantities for both reaction classes below T_c are large and positive (Table II). The fractions of the total susceptible bonds cleaved in each reaction at various temperatures are compiled in Table I. Below T_c these percentages are essentially independent of temperature; average values of 16 ± 3% (fast reaction) and 84 ± 4% (slow reaction) may be calculated. It should be noted that this ratio of bonds split in the fast to those split in the slow reaction is very close to that found for ichthyocol gelatin recooled below T_c; where values of 18 ± 4% (fast reaction) and 82 ± 4% (slow reaction), also apparently invariant with temperature, were obtained.[25] The implications of these various data with respect to the molecular structure of the collagen macromolecule will be considered in the General Discussion.

It is important to demonstrate that all the reactions observed by means of the pH-stat represent the actual cleavage of peptide bonds, rather than the liberation of protons as a consequence of some "unmasking" of side-chain residues. Two independent lines of evidence lead to this conclusion: (1) since changes measured by the colorimetric ninhydrin method must be due to the appearance of new α-amino groups, the close agreement mentioned above between parallel ninhydrin and pH-stat runs, both with respect to apparent rate constants and the fraction of bonds split in each reaction, indicates that only peptide bond cleavage is being measured by the pH-stat and (2) the apparent pK (pK') of the ionizable groups released is close to that expected for free α-amino groups in polypeptide chains.

Values of pK' were obtained in several ways: (1) P_∞ was measured in a series of pH-stat runs at various pH values. These data, obtained using gelatin at 30°, are presented in the lower portion of Fig. 3. The solid curve was derived by calculation, based on the averages of four or five determinations each of P_∞ at pH 8.0 and 8.3 (solid points). These lead to a pK' (at 30°) of 8.13, which results in a pK'_{25} of 8.29 when corrected to 25° by the equation of Harned and Robinson.[37,38] The experimental points obtained directly are in reasonable agreement with this value of pK' though displaced downward slightly at high pH, and upward somewhat at the lower pH values (Fig. 3). These deviations might be attributed to a slight "polydispersity" in pK'. (2) An approximate value of pK' was derived from differential titrations of completely digested and undigested samples of ichthyocol at approximately 30°. The results are shown in the insert, Fig. 3. Clearly the experimental points fit quite well onto the curve calculated for pK' = 8.1 (3) pK' was also obtained by direct comparison of N (total number of α-amino groups released enzymatically) determined by the colorimetric ninhydrin technique, with P_∞ from parallel pH-stat runs. An average pK'_{25} of 8.15 resulted from these measurements. Thus it appears that an average pK'_{25} of 8.2 ± 0.1 may be assigned to the collagenase-liberated α-amino groups.

It may be noted that this pK'_{25}, while well within the range expected of α-amino groups in polypeptide chains, is somewhat higher than the usual average value (7.6 – 7.9).[39] This result offers an interesting, though indirect, confirmation of the amino acid specificity proposed for collagenase[16–19] (collagenolytic attack seems to liberate mostly N-terminal gly.pro-) since the $pK'_{25}(NH_3^+)$ values tabulated by Cohn and Edsall[38] for a series of dipeptides place that for gly.pro at 8.53, well above the average value of 8.24 for the dipeptides listed.[40]

(36) H. Neurath and G. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(37) H. S. Harned and R. A. Robinson, *Trans. Faraday Soc.*, **36**, 973 (1940).

(38) E. J. Cohn and J. T. Edsall, "Proteins and Amino Acids," Reinhold Publishing Co., New York, N. Y., 1943, Chap. 4.

(39) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, Chap. 9.

(40) J. P. Greenstein[41] has also pointed out that the –CONH– peptide linkage (as in glycylglycine) increases the acidity of an adjacent amino group, compared to the free amino acid, considerably more than the –CON< peptide linkage (*e.g.*, glycylproline).

We have qualitatively confirmed this general tendency, for N-terminal glycine compounds, by titrating the α-amino group of a series of N-terminal glycine peptides at about 30° in 0.17 M $CaCl_2$. The uncorrected pK' (NH_3^+) values estimated from the titration curves for the various peptides (to ±0.1 pH units) are given in parentheses:

II. Macromolecular Size and Shape Changes.—Having examined directly the time course of the proteolysis of collagen by collagenase, we now turn to a comparative study of the molecular changes which accompany this degradation. In this portion, the structural alterations reflected by changes in viscosity, light-scattering, non-dialyzable protein concentration and optical rotation will be taken up in turn. First, however, the light-scattering and viscosity behavior of undegraded ichthyocol in neutral salt solution must be briefly considered.

Molecular Weight and Configuration of Soluble Ichthyocol in 0.5 *M* $CaCl_2$.—As Boedtker and Doty[9] have pointed out, and as our remarks (see "Materials and Methods") on preparing solutions of ichthyocol for light-scattering substantiate, reproducible light-scattering measurements on this material are difficult to achieve. As a consequence, different investigators using this technique have reported very diverse values for the molecular weight of soluble collagen (see ref. 9). Therefore, before beginning studies on the collagenolytic degradation of ichthyocol collagen, we examined the light-scattering properties of ichthyocol in the absence of the enzyme. A series of preparations of ichthyocol, dissolved in *p*H 7.0, 0.5 *M* $CaCl_2$–0.05 *M* Tris buffer, were subjected to the clarification procedures described above; scattered intensities were measured (5°C.), complete Zimm plots were constructed and weight average molecular weights and Z-average radii of gyration calculated. These values for a series of experiments are compiled in Table III and an example of the Zimm plots from which they were derived is presented in Fig. 4. (Note that in Fig. 4, scattering is essentially independent of protein concentration over this range and that there is no apparent residual curvature at the low angle points—the latter would be indicative of the presence of large particles of "dust".[9])

TABLE III

MOLECULAR WEIGHTS AND RADII OF GYRATION FOR SEVERAL PREPARATIONS OF ICHTHYOCOL[a]

$\bar{M}_w$	$\bar{r}_g$ (Å.)
1.00×10^6	1.58×10^3
0.89×10^6	1.28×10^3
1.75×10^6	1.58×10^3
1.37×10^6	1.57×10^3
1.92×10^6	1.64×10^3

[a] 0.5 *M* $CaCl_2$–0.05 *M* Tris, *p*H 7.0.

Table III shows considerable scatter in weight average molecular weight ($\bar{M}_w$) from one preparation to another ($\bar{M}_w$, av. = $1.39 \pm 0.38 \times 10^6$, $\bar{r}_g$, av. = $1.52 \pm 0.12 \times 10^3$ Å.) and also that the values obtained are considerably higher than those measured by Boedtker and Doty[9] in *p*H 3.7 citrate buffer, ($\bar{M}_w$, av. = 345,000, $\bar{r}_g$, av. = 870 Å.). Because of this discrepancy and in order to further insure that "dust" was not influencing the result despite all precautions, several samples of ichthyocol dissolved in 0.1 *M* sodium citate at *p*H 3.7 were prepared and carried through the clarification procedures *together with* samples in neutral (0.5 *M* $CaCl_2$) solution. The weight average molecular weights obtained in citrate buffer were in reasonable agreement with those of Boedtker and Doty ($\bar{M}_w$ = 4–5 × 10^5) and always a factor of two or more lower than the values of $\bar{M}_w$ obtained from the corresponding samples in neutral solution. Thus we conclude that the molecular weight difference observed is a real one and that probably ichthyocol in neutral solution exists as rather polydisperse citrate "monomer" aggregates of small degree of polymerization. This conclusion is reinforced by the apparent average particle shape, as revealed by a plot of reciprocal particle scattering factor ($P(\theta)^{-1}$) *versus* angle (lower experimental curve, Fig. 10). The ichthyocol "monomer" behaves very much like a rigid rod in such a plot,[9] while the aggregates in neutral solution appear to deviate toward a more "coil-like" configuration indicative of relatively random polymerization (Fig. 10).

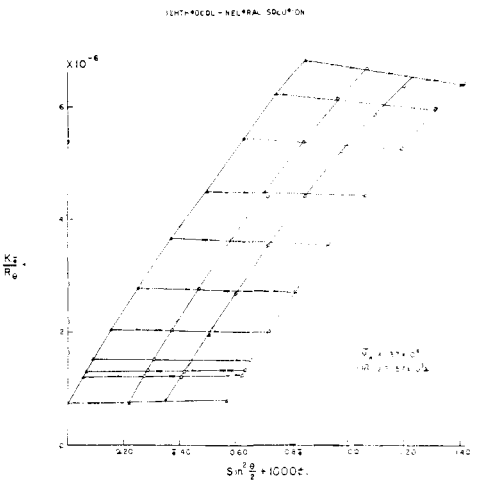

Fig. 4.—Zimm plot for neutral ichthyocol in 0.5 *M* $CaCl_2$–0.05 *M* Tris, *p*H 7.0; *T* 5°; $\bar{M}_w = 1.37 \times 10^6$; $\bar{r}_g = 1.57 \times 10^3$ Å. Protein concentrations are: 0.22, 0.35 and 0.57 mg./ml.

The intrinsic viscosity [η] of ichthyocol has been determined in both citrate buffer at *p*H 3.7 and in neutral solutions. In citrate, [η] = 10–14 dl./g.[6,9] while in neutral salt values of 12–16 dl./g.[23] have been obtained. (The increased intrinsic viscosity measured at neutral *p*H is presumably due to the small aggregates observed by light-scattering.) These high values of intrinsic viscosity have been attributed to the rod-like character of the multistranded collagen macromolecule, since [η] drops to ~0.4 dl./g.,[7,11,25] about 3% of the original value, on conversion to presumably single-chain, randomly coiled parent gelatin.

Since the ichthyocol "monomer" appears to be a rigid particle and since the Ostwald viscometers used in most of the present studies exhibit a high shear gradient ($\beta_{av} \simeq 1400$ sec.$^{-1}$ for water at 20°), the consequences of possible non-Newtonian flow must be considered. Utilizing the molecular weight and dimensions of the ichthyocol "monomer" and tables of intrinsic viscosity for prolate ellipsoids as a function of shear gradient,[42] it can

glycylglycine (8.1), glycylglycylglycine (7.9), tetraglycine (7.8), glycyl-L-phenylalanine (8.1) glycyl-L-glutamic acid (8.2), glycyl-L-proline (8.3) and glycyl-L-hydroxyproline (8.3.).

(41) J. P. Greenstein, *J. Biol. Chem.*, **101**, 603 (1933).

(42) H. A. Scheraga, *J. Chem. Phys.*, **23**, 1526 (1955).

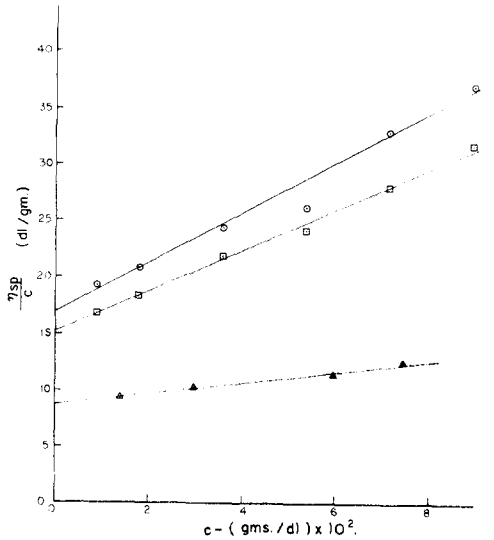

Fig. 5.—Reduced viscosity of neutral ichthyocol at various shear gradients. (⊙), T = 15.65°, β_b (for 0.5 M $CaCl_2$ at temp. of experiment) = 150 sec.$^{-1}$, $[\eta]$ = 17.0 ± 0.5 dl./g. (⊡), T = 15.65°, β_b = 1100 sec.$^{-1}$, $[\eta]$ = 15.3 ± 0.2 dl./g. (△), Ichthyocol partially degraded by collagenase—enzyme inactivated with 0.072 M cysteine (see text). T = 19.45°, β_b = 1200 sec.$^{-1}$, $[\eta]$ = 8.7 ± 0.2 dl./g.

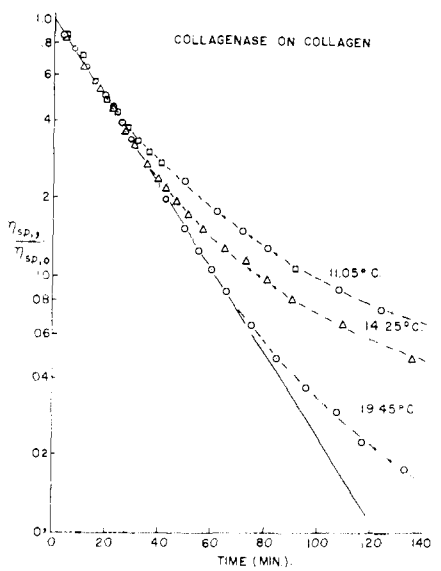

Fig. 6.—Fractional specific viscosity (logarithmic scale) of ichthyocol as a function of time after adding collagenase. (⊡), 11.05°; (△), 14.25°; (⊙), 19.45°. All data normalized to same original slope (solid line).

be shown that $[\eta]_{\beta = 1400 \text{ sec.}^{-1}}$ is only underestimated by 5% in comparison with $[\eta]_{\beta = 0}$ for this particle. This calculation is in agreement with the finding that $[\eta]$ for ichthyocol in pH 3.7 citrate is essentially independent of shear gradient ($\beta \leq$ approx. 1400 sec.$^{-1}$) at low protein concentrations.[9] On the other hand, for the larger particles observed in neutral solution by light-scattering, the orienting effect (and thus the effect on $[\eta]$) of the large shear gradients in the viscometers used might be considerably greater—to an extent depending on the average shape of the aggregates.

Yang has recently shown, using poly-γ-benzyl-L-glutamate in various solvents, that rigid particles and random coils can be readily differentiated on the basis of their non-Newtonian behavior. He found, in agreement with theory,[42] that under high shearing stress the intrinsic viscosity of rigid rods decreases to less than one-tenth of its value at $\beta = 0$, while shear gradients of the same magnitude lower $[\eta]$ for random coils of similar molecular weight much less.[43,44] Light-scattering has suggested that ichthyocol in neutral solution forms aggregates approaching a "coil-like" configuration (see Fig. 10 and above). The intrinsic viscosity of such particles should exhibit shear dependence of the same order of magnitude as that found for the ichthyocol "monomer." If, on the other hand, the increased values of $[\eta]$ and $\bar{M}_w$ measured in neutral solution correspond to *rigid* aggregates of about four "monomers," then, substituting the axial ratio calculated using Simha's equation[45] and $\bar{M}_w$ into Scheraga's tables,[42] we may compute that $[\eta]$ measured at β = 1400 sec.$^{-1}$ should be underestimated by approximately *45%*. On this basis, the average value of the intrinsic viscosity for ichthyocol in neutral solution ($[\eta] \simeq$ 15 dl./g.), measured in high gradient Ostwald viscometers, would correspond to $[\eta]_{\beta = 0}$ of ~27 dl./g.

To examine this possibility we determined the intrinsic viscosity of neutral ichthyocol at β = 1100 and 150 sec.$^{-1}$. The results are shown in the upper two lines of Fig. 5; values of $[\eta]_{\beta = 150 \text{ sec.}^{-1}}$ = 17.0 ± 0.5 dl./g., and $[\eta]_{\beta = 1100 \text{ sec.}^{-1}}$ = 15.3 ± 0.2 dl./g. were obtained. (For comparison, data on a sample of ichthyocol, about 50% degraded by collagenase as determined by viscometric assay, are included in Fig. 5.) These results indicate that the "monomers" in neutral solution are *not* rigidly aggregated, in agreement with the conclusions drawn from light-scattering. Also it would appear that the degradation studies presented below will not be appreciably perturbed by non-Newtonian behavior of the particles in the high-gradient viscometers during the early stages of protolysis.

Viscometry.—The high viscosity of ichthyocol solutions makes viscometry a useful technique for following the proteolytic degradation of the collagen macromolecule. In earlier work it was noted that the logarithm of the specific viscosity of an ichthyocol solution falls linearly with time after adding collagenase; indeed, this behavior forms the basis of the viscometric assay for collagenase.[15] In Fig. 6, the fractional specific viscosity ($\eta_{sp,t}/\eta_{sp,0}$) of ichthyocol at three temperatures is plotted on a logarithmic scale against time following collagenase addition. (The specific viscosity of ichthyocol solutions when proteolysis has gone to completion ($\eta_{sp,\infty}$) is less than 1% of $\eta_{sp,0}$ and thus is not considered.) It is apparent that the experimental points do fall on a straight-line for a considerable part of the reaction (1.5 to 4 half-lives, depending upon temperature). Note that deviations from linearity arise earlier at the lower temperatures—this point will be commented on below.

(43) J. T. Yang, THIS JOURNAL, **80**, 1783 (1958).
(44) J. T. Yang, *ibid.*, **81**, 3902 (1959).
(45) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

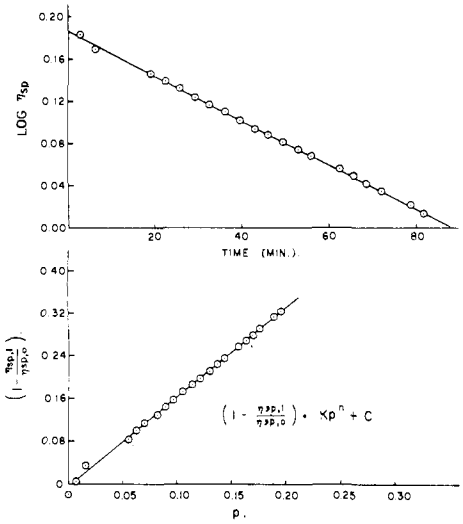

Fig. 7.—(a) Logarithm of the fractional specific viscosity of ichthyocol as a function of time after adding collagenase. 14.2°, 0.54 mg./ml. collagen, 0.19 units/ml. collagenase. (b) Fractional specific viscosity decrease as a function of p, the probability that a given susceptible peptide bond has been split. Viscosity data from Fig. 7a, p from parallel colorimetric ninhydrin determinations.

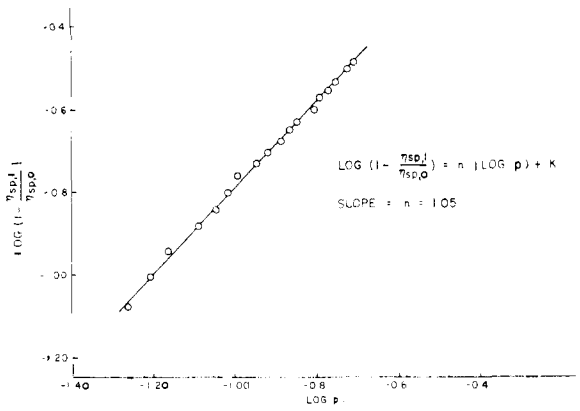

Fig. 7c.—Logarithm of fractional specific viscosity decrease as a function of log p. Data from Fig. 7b. Slope, $n = 1.05$.

Figure 7a is a similar plot, of the early part of the reaction, for an experiment in which a very low enzyme concentration was employed to "stretch out" the primary phase. Again the linear (logarithmic) nature of the specific viscosity fall is confirmed; also Fig. 7a shows quite clearly the total absence of an initial lag period. Since peptide bond cleavage has also been shown (Fig. 1) to obey essentially first order kinetics,[46] it would appear that the decrease in η_{sp} must be directly related to some power of the number of peptide bonds split. Fig. 7b, in which the data presented in Fig. 7a are replotted as $1 - (\eta_{sp,t}/\eta_{sp,0})$ *versus* p (p is defined as the probability of splitting a give susceptible peptide bond and goes from 0 to 1 as proteolysis proceeds), shows this to be the case; a straight line results up to at least $p = 0.2$.

Thomas[47] and Schumaker, Richards and Schachman[48] have examined DNA by various physicochemical methods during desoxyribonuclease-catalyzed degradation and have utilized the results to draw certain conclusions about the structure of the substrate. Schumaker, *et al.*, in particular, examined theoretically the viscometric consequences of random single-chain scissions of a multi-stranded molecule and derived the following expression (applicable during the early portion of the enzymatic reaction) relating η_{sp} and p

$$\log [1 - (\eta_{sp,t}/\eta_{sp,0})] = n \log p + K \qquad (1)$$

where n is the number of strands in the macromolecule and K is an arbitrary constant. We have attempted to apply this equation to the enzymatic degradation of ichthyocol.

If one plots $\log [1 - (\eta_{sp,t}/\eta_{sp,0})]$ *versus* $\log p$, n is the slope of the resulting straight line. Such a plot for the ichthyocol–collagenase system is shown in Fig. 7c; a value of $n = 1.05$ was obtained. Thus, within the limits of error, n for this system is approximately unity. (That n must be close to unity is also evident from the linearity of Fig. 7b.) Both this result and the lack of an initial lag period in plots such as Figs. 6 and 7a indicate that the decrease in specific viscosity per enzymatic "hit" is a constant during this portion of the reaction and therefore, according to the theory of Schumaker, *et al.*, that the collagen macromolecule is a single-stranded structure — at least in the regions around the collagenase-susceptible bonds. However, this interpretation is contrary to all the previous X-ray[49–51] and physicochemical[6,9] evidence, which strongly favors a three-stranded structure, particularly in glycine- and proline-containing portions of the polypeptide chains.

Schumaker, Richards and Schachman themselves suggest several paths out of this dilemma. As they point out, equation 1 is based on several assumptions: (1) that the strands are continuous throughout the substrate macromolecule, (2) that the enzyme cleaves only a single-strand in each attack and (3) that the cleavage of a single-strand in a multi-stranded region does *not* introduce additional flexibility into the macromolecule. If any of these assumptions are unjustified, the effect is manifested as a decrease in the value of n from its theoretical value. In the case of ichthyocol, the evidence to be developed below indicates that collagenase *does* split single-strands *in multi-stranded regions* and that the direct dependence of η_{sp} upon p results from the breakdown of assumption 3 — that is, each enzymatic "hit" *does* introduce an additional increment of flexibility into the macromolecule. This flexibility increment need not be very large to account for the data — for as Schu-

(46) Actually, during the early part of the reaction the pH-stat kinetics have been interpreted as the sum of two first order reaction classes (see above). However, because the fast reaction is so much more rapid than the slow, almost all of the cleavage observed during this portion may be attributed to the former.

(47) C. A. Thomas, Jr., THIS JOURNAL, **78**, 1861 (1956).

(48) V. N. Schumaker, E. G. Richards and H. K. Schachman, *ibid.*, **78**, 4230 (1956).

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

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

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

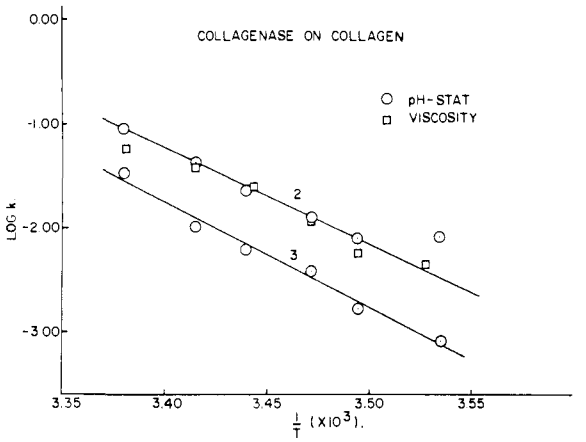

Fig. 8.—Arrhenius plots, collagenase on ichthyocol. (⊙), *p*H-stat data, from Fig. 2, reactions 2 and 3. (⊡), Viscosity data (see text).

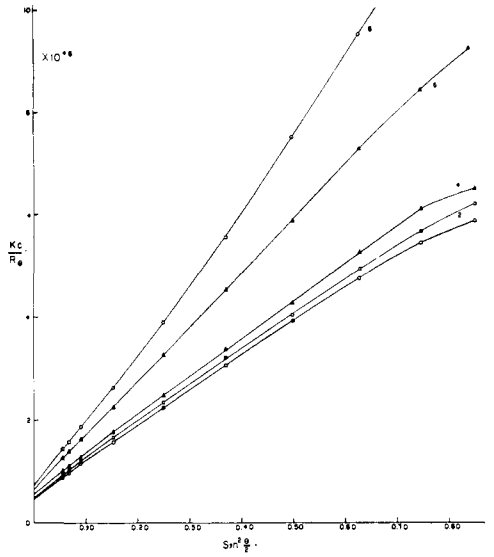

Fig. 9.—Zimm plots obtained at various times during the proteolysis of ichthyocol by collagenase. 14.5°, initial ichthyocol concentration = 0.80 mg./ml., 0.87 units/ml. collagenase. (1) Before enzyme; (2) 3.9 min. after adding enzyme; (4) 32.0 min.; (6) 206 min.; (8) 3370 min. (See text and Table IV.)

maker, *et al.*, have shown, a very small increase in flexibility per "hit" results in a very substantial decrease in n. Parenthetically, attributing the viscosity fall as a consequence of enzyme action primarily to increased macromolecular flexibility also serves to explain the effect of temperature seen in the experimental curves of Fig. 6. Thus one might well expect the (enzymatic) insertion of potential points of flexure along the macromolecule to be more effective in reducing the viscosity at higher temperatures, while the viscosity decrease accompanying the simple cleavage of a rigid rod into two shorter rigid rods should not be noticeably temperature dependent.

In order to correlate further the viscometric effect of collagenase on collagen with the actual cleavage of peptide bonds as measured directly by the *p*H-stat or ninhydrin techniques, we examined the effect of temperature on k (the apparent first

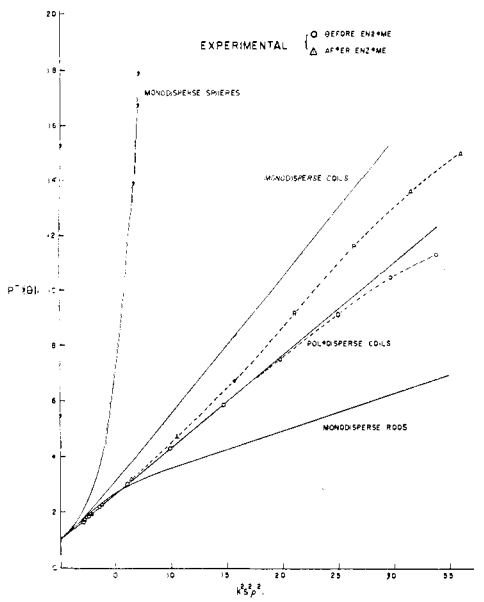

Fig. 10.—Comparison of normalized Zimm plots on ichthyocol before (⊙) and after (⊙) collagenolytic degradation (data from Fig. 9 runs (1) and (8)) with theoretical curves for various particle shapes.

order rate constant) derived from viscosity measurements. Values of k were obtained at various temperatures from the linear portions of curves such as those of Fig. 4, normalized with respect to enzyme and initial substrate concentrations as above and plotted as log k' *versus* $1/T$. In Fig. 8, these points have been superimposed on the low temperature portion of Fig. 2, in order to afford direct comparison. It would appear that the viscometric points fall, with reasonable precision, on the line drawn through the data for the fast reaction measured with the *p*H-stat, suggesting that the early viscometric changes reflect primarily the progress of this reaction.

Light-Scattering.—The degradation of ichthyocol by collagenase was also followed by light-scattering; Table IV summarizes the results of a typical experiment. Column 2 shows that the weight average molecular weight falls by about 25% during the entire degradation, while column 3 reveals that $\bar{r}_g$ is not greatly changed. Certain of the Zimm plots used to calculate these values are presented in Fig. 9.

It is particularly interesting to observe (Fig. 9) the change in shape of the over-all Zimm plot during proteolysis. Thus while the original curve appeared concave downward, as digestion proceeds the curves became linear and then concave *upward*. This type of behavior was observed consistently in every experiment and seemed to suggest a change in macromolecular shape occurring as a consequence of proteolysis. To examine this possibility, the data obtained: (1) before enzyme addition and (2) after 3370 minutes of proteolysis, are compared (after adjustment to a common molecular weight and radius of gyration) with theoretical curves for models of various shapes in Fig. 10. (Here $P(\theta)^{-1}$ is the reciprocal particle scattering factor, $k^2s^2\rho^2$ is a function of radius of gyration and

TABLE IV

LIGHT-SCATTERING MEASUREMENTS ON COLLAGENASE ICHTHYOCOL[a]

Time (min.)[b]	Mol. wt. (av.)	$\bar{r}_g$ (Å.)
Before enzyme	1.92×10^6	1.64×10^3
3.9	1.82×10^6	1.63×10^3
12.5	1.64×10^6	1.53×10^3
32.0	1.64×10^6	1.58×10^3
113	1.30×10^6	1.51×10^3
206	1.39×10^6	1.70×10^3
360	1.28×10^6	1.63×10^3
3370	1.22×10^6	1.69×10^3

[a] Incubated at 14.5°. Used 0.80 mg./ml. ichthyocol, at zero-time added 0.87 units/ml. of collagenase. [b] Time after enzyme.

$\sin \frac{\theta}{2}$, and the experimental data were adjusted to an intercept of unity and an initial slope of $1/3$.) Clearly proteolysis has resulted in an over-all elevation of the $P(\theta)^{-1}$ *versus* $k^2s^2\rho^2$ curve, away from the theoretical curve for rods and toward that for monodisperse random coils.

There are at least three possible explanations of such an elevation of the reciprocal particle scattering curve—unattended by a parallel increase in molecular weight. These are: (1) a *decrease* in polydispersity, (2) a considerable increase in branching and (3) conversion to a more flexible structure. We now proceed to a more detailed consideration of these alternatives.

(1) Benoit[52] has shown that increasing the polydispersity of a solution of random coils results in an over-all lowering of the $P(\theta)^{-1}$ curve. Goldstein[53] demonstrated that similar considerations apply to polydisperse systems of rigid rods, and Rice[54] proved that this is so in the general case. Thus a *rise* in the $P(\theta)^{-1}$ plot could, in theory, be attributed to a *decrease* in polydispersity. However, such an effect could only be produced by collagenase *via* a general "homogenization," which of necessity would be accompanied by a *much* larger decrease in $\bar{M}_w$ than is observed experimentally.

(2) Benoit[52] also showed that a rise in the $P(\theta)^{-1}$ curve for random coils can come about as a result of "branching." Such behavior is not, *a priori*, unlikely to be exhibited during the course of proteolysis of a multi-stranded macromolecule, since one might expect some "fraying-out" of cleaved single strands to be initiated at enzymatically produced breaks in the polypeptide chains as a consequence of local hydrogen bond breaking or "unzippering."

(3) Peterlin[55] has calculated the effect of introducing additional points of flexure into initially rigid linear macromolecules. He showed that as the "persistence length" is decreased from the length of the entire macromolecule (rigid rod) to zero (random coil), the entire $P(\theta)^{-1}$ curve is elevated correspondingly. Therefore increased macromolecular flexibility alone can also bring about a considerable rise in the reciprocal particle scattering factor curve.

(52) H. Benoit, *J. Polymer Sci.*, **11**, 507 (1953).
(53) M. Goldstein, *J. Chem. Phys.*, **21**, 1255 (1953).
(54) S. A. Rice, *J. Polymer Sci.*, **16**, 94 (1955).
(55) A. Peterlin, *ibid.*, **10**, 425 (1953).

It thus appears that an increase in either "branching" or "flexibility" or both can account for the *direction* of the change in the $P(\theta)^{-1}$ curve. However, the explanation adopted must also be compatible with both the observed changes in $\bar{r}_g$ (light-scattering) and η_{sp} which accompany collagenolysis. As shown above, η_{sp} falls exponentially (to less than 10% of its initial value) during the early stages of digestion, while $\bar{r}_g$ remains approximately constant (Table IV). These facts, taken together, are incompatible with either branching or flexibility changes alone.

Zimm and Stockmayer[56] have shown theoretically that introducing branches into *random coils* at constant molecular weight brings about a moderate *decrease* (<30%) in the radius of gyration and intrinsic viscosity. This has been confirmed experimentally by several groups using cross-linked polystyrene[57] and variously branched dextrans.[58,59] On the other hand, it can be easily shown (see Appendix) that introducing branches at constant molecular weight into *rigid rods* generally results in an *increased* r_g (and perhaps $[\eta]$). Thus branching *via* a "fraying-out" of cleaved single-strands of the relatively rigid ichthyocol macromolecule, *without a concomitant increase in flexibility*, might be expected to lead to a moderate *increase* in $\bar{r}_g$.

As pointed out above, an increase in macromolecular flexibility alone can account for the fall in η_{sp} during digestion. However, a simple increase in flexibility should be accompanied by a marked *decrease* in $\bar{r}_g$ as the random coil configuration is approached at constant molecular weight. Thus an increase in flexibility alone is not compatible with the relative constancy of $\bar{r}_g$ observed by light-scattering. Therefore it appears that flexibility and branching are probably both involved in the observed changes, though flexibility must play the dominant role viscometrically; (see also the non-dialyzable protein results below).

Non-Dialyzable Protein.—In order to confirm this interpretation of the effect of collagenase on the collagen macromolecule, we examined the rate of disappearance of non-dialyzable protein during proteolysis. Fig. 11 presents the results of an experiment in which the decrease in specific viscosity, 90° light-scattering and non-dialyzable protein were all monitored simultaneously during collagenolysis. The relative rates of fall of specific viscosity and light-scattering, and the final levels attained, were close to those expected on the basis of the previous results; (though it should be pointed out that 90° scattering does not constitute a direct measure of $\bar{M}_w$ because of the large dissymmetry shown by solutions of soluble collagen). On the other hand, the observed decrease in non-dialyzable protein to about 30% of the original value is *not* compatible with previous findings. Further investigation showed that this

(56) B. H. Zimm and W. H. Stockmayer, *J. Chem. Phys.*, **17**, 1301 (1949).
(57) C. D. Thurmond and B. H. Zimm, *J. Polymer Sci.*, **8**, 477 (1952).
(58) M. Wales, P. A. Marshall, S. Rothman and S. G. Weissberg, *Ann. N. Y. Acad. Sci.*, **57**, 353 (1953).
(59) K. A. Granath, *J. Colloid Sci.*, **13**, 308 (1958).

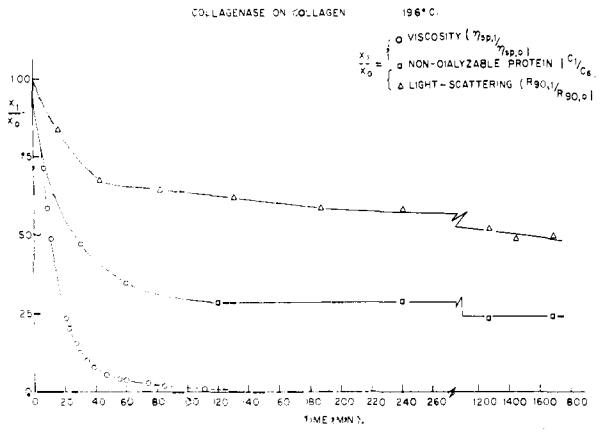

Fig. 11.—Fractional decrease in 90° light-scattering intensity, (△); non-dialyzable protein concentration, (⊡); and specific viscosity, (⊙) as a function of time after adding collagenase. 19.6°, 0.40 mg./ml. ichthyocol, 0.45 units/ml. collagenase.

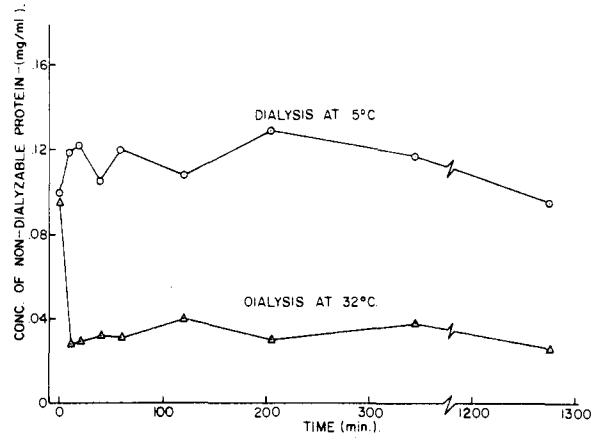

Fig. 12.—Concentration of non-dialyzable protein as a function of time after adding collagenase. At times indicated, enzyme inactivated with 0.45 *M* cysteine. Dialysis at 5°, (△); and 32°, (⊙). Reaction at 13.1°, 1.27 mg./ml. ichthyocol and 2.5 units/ml. collagenase.

apparent discrepancy is due to the method used to inactivate the enzyme prior to dialysis.

As pointed out in the Introduction, heating a solution of collagen to temperatures above T_c results in the dissociation of the multi-stranded collagen into single-chain gelatin. Below T_c these strands are presumably held together by inter-chain hydrogen bonds[9,50] which rupture during the conversion to gelatin. Since the final average molecular weight of enzymatically degraded collagen is about 500, *per N-terminal α-amino group* (or *C*-terminal carboxyl),[16,17] the light-scattering results seem to require that most of these small pieces be held together (by means of the original inter-chain hydrogen bonds) in order to maintain the particle weight at the observed level. Therefore conditions tending to rupture these hydrogen bonds would be expected to lead to a considerable decrease in non-dialyzable protein. In the experiment presented in Fig. 11, aliquots of the digestion mixture were removed at various times after adding collagenase and the enzyme inactivated by heating to 70° for 15 minutes prior to dialysis

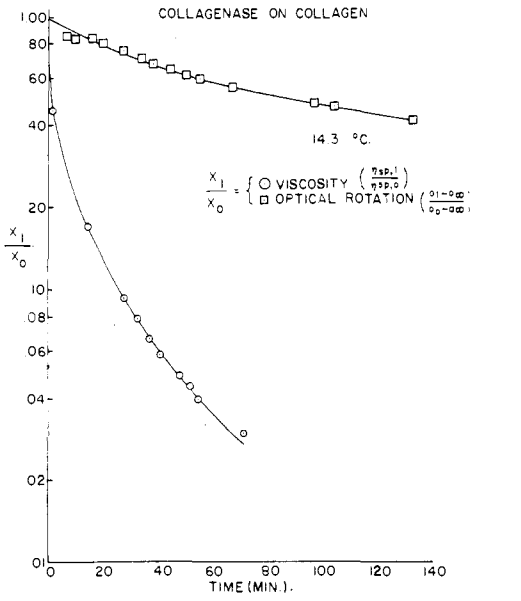

Fig. 13.—Fractional decrease in optical rotation, (⊡); and specific viscosity, (⊙); as a function of time after adding collagenase. 14.3°, 2.0 mg./ml. ichthyocol and 8.3 units/ml. collagenase.

in the cold. This treatment might be expected to rupture the hydrogen bonds connecting the residual chain fragments and to release most of the protein into solution as small, freely dialyzable peptides. This interpretation of the above discrepancy was confirmed by the experiment illustrated in Fig. 12.

Here an ichthyocol solution was subjected to collagenolytic attack at 13.1° and duplicate aliquots of the digestion mixture were removed at various times after adding the enzyme, but this time collagenase was inactivated, *in the cold*, with 0.45 *M* cysteine. The duplicate sets of aliquots were then dialyzed at two different temperatures: one set at 5° and the other at 32°—the latter well above T_c for the collagen → gelatin conversion. Note that the non-dialyzable protein concentration of the samples dialyzed at the higher temperature again fell to about 30% of the zero-time value. However, as might be expected on the basis of the reasoning outlined above, the 5° samples released essentially no dialyzable material. The results at the higher temperature also indicate that some (presumably covalently-bonded) fragments of collagen with molecular weight in excess of 5–10 × 10^3 remain even after collagenolytic degradation has gone to completion, suggesting that the initial distribution of collagenase-susceptible peptide bonds along the collagen macromolecule might be relatively non-random.

Optical Rotation.—Figure 13 presents the results of an experiment in which the fall in specific viscosity ($\eta_{sp,t}/\eta_{sp,0}$) and optical rotation $\left(\frac{\alpha_t - \alpha_\infty}{\alpha_0 - \alpha_\infty}\right)$ during collagenolytic degradation were monitored concurrently. Fig. 13 re-emphasizes, as has been pointed out previously,[60] that the specific viscosity decreases much more rapidly than the optical ro-

(60) S. Seifter, P. M. Gallop and E. Meilman, in "Recent Advances in Gelatin and Glue Research," G. Stainsby, Ed., Pergamon Press, London, 1957, pp. 164–169.

tation during proteolysis. In terms of the above data, this seems to indicate that the collagen macromolecule not only remains relatively intact in terms of particle size (see light-scattering and non-dialyzable protein concentration data) but also in over-all helical content. These results, furthermore, are in marked contrast to data obtained during the conversion of collagen to gelatin, where the viscosity and the rotation fall together as the temperature is increased.[35]

General Discussion

In a recent preliminary note, Harrington, von Hippel and Mihalyi[24] presented some observations on the kinetics of proteolysis of several fibrous proteins, noting that in each case the over-all kinetics could be reduced to the sum of two concurrent, apparent first order reactions proceeding at markedly differing rates. It was also pointed out that denaturation appeared to bring about the transfer of some susceptible bonds from one reaction class to another (myosin–trypsin), and in the more favorable case of gelatin acted upon by collagenase, actually seemed to transfer all the bonds to a single reaction class exhibiting the thermodynamic parameters usually associated with the cleavage of synthetic substrates and denatured proteins. This fact seemed to suggest that the rate of enzymatic hydrolysis of the various susceptible peptide bonds depends more on the configurational (secondary structure) than on the chemical (amino acid) environment of the bonds. Since these studies were carried out on fibrous proteins (which, by definition, differ from globular proteins in that they have no complicating "tertiary" structure)[61] the simplest interpretation of the two reaction classes—reduced to one on complete denaturation—seemed to be that the enzyme differentiates between "amorphous" and "crystalline" (in an X-ray diffraction sense) regions along the polypeptide chains.

The systems upon which the above conclusions were based have since been examined much more extensively. The results obtained with collagenase on collagen are reported here—the myosin–trypsin and the gelatin–collagenase studies have been discussed in detail elsewhere.[25,62] While the above "amorphous-crystalline" interpretation still seems to be the most reasonable for the myosin–trypsin case, the collagen–gelatin–collagenase situation appears to be more complicated. In particular, the gelatin–collagenase studies cited above,[25] in which the proteolysis of ichthyocol gelatin above and below the gelatin→collagen-fold transition temperature was examined, offer some additional insight into the features of substrate configuration to which collagenase is sensitive.

It is well-known that mild heating transforms collagen into single-chain, essentially randomly coiled parent gelatin. It has also been shown that this transformation is, to a considerable extent, reversible; since a collagen-like configuration of the polypeptide chains is at least partially restored on recooling to temperatures below T_c.[8,25] However, the redevelopment of collagen-like structure after recooling is relatively slow, making it possible to examine the kinetics of proteolysis of recooled gelatin solutions before either helical configuration (detected by optical rotatory changes) or inter-chain associations (followed by viscosity and light-scattering) have developed to any appreciable extent. It was found in such experiments that the complex kinetics of proteolysis (two reactions) seemed to be restored immediately after cooling, long before the restoration of the over-all structure. Thus the enzyme, though sensitive to configuration, did not appear to be sensitive to the over-all polypeptide chain configuration as previously assumed. By reasoning from the behavior of proline-containing synthetic polypeptides and the amino acid sequence specificity of collagenase, it was concluded that the enzyme is actually sensitive to the appearance of *local* precursors of the final structure and, in particular, to a *local intra-chain configurational change* involving the locking of proline (and hydroxy-proline) residues into the poly-L-proline II configuration.[25]

Since the proteolysis of soluble collagen, as detailed in this communication, resembles so closely the proteolysis of gelatin at temperatures below T_c, we will assume that the arguments developed previously on the basis of the gelatin work[25] apply to collagen as well. However, it should be noted that though the ratio of susceptible bonds cleaved in the two reaction classes is about the same for "cold" gelatin as for unheated collagen (see Results section), the apparent enthalpy and entropy of activation of proteolysis for the two systems differ markedly. Whereas, after initial "gelatinization," values of $\Delta H^{\ddagger}$ of about +22 and +29 kcal./mole and of $\Delta S^{\ddagger}$ of approximately +30 and +51 e.u. were obtained for the two reaction classes in "cold" gelatin, the corresponding quantities obtained with unheated ichthyocol are considerably larger (see Table II). If we assume, as in the "cold" gelatin case, that the enzyme distributes the susceptible bonds into the two observed reaction classes on the basis of *local* configuration,[25] then the uniformly higher ΔH^{*} (about +20 kcal./mole) and $\Delta S^{\ddagger}$ (about +60 e.u.) found for each class with unheated collagen might well be a measure of the "tighter" folding of the polypeptide chains in the collagen macromolecule—superimposed on the effect of local configurational differences.[63]

Turning now to the effect of collagenolytic peptide bond cleavage on the structure of the collagen macromolecule, the following picture emerges

(61) The usage of the terms "secondary" and "tertiary" structure has varied from one author to another. In this and previous papers[24,25] we have arbitrarily applied the term "secondary" structure to all aspects of the configuration of one or more parallel polypeptide chains in which the *over-all* (or average) chain direction remains unchanged (as in fibrous proteins) and reserve "tertiary" structure for the larger scale configurational features, such as chain reversal and the packing together of ordered and disordered regions, characteristic of the compact globular protein molecules.

(62) E. Mihalyi and W. F. Harrington, *Biochim. Biophys. Acta*, **36**, 447 (1959).

(63) That "cold" gelatin forms a "looser" or "more imperfect" structure than the parent collagen is borne out both by the significantly lower final specific levorotation of recooled gelatin[8] and by the fact that gelatin solutions of sufficient concentration undergo gelation in the cold.

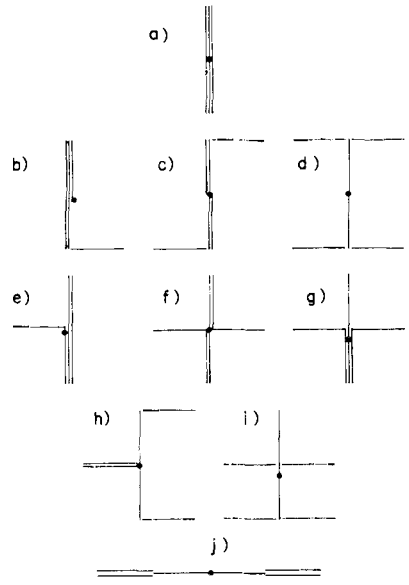

Fig. 14.—Two dimensional, schematic models of branched forms of a 3-stranded, rigid rod (Fig. 14a); (●), locates the center of mass of each model, about which the radius of gyration is calculated (Table V).

from the results presented above. It appears that ichthyocol, in neutral salt solution, exists mostly as small degree of polymerization aggregates of the rigid three-stranded citrate "monomer." Collagenase seems to attack these structures mostly by catalyzing single-chain scissions, making the macromolecule more flexible by introducing new points at which it can bend but leaving the particle weight relatively unaltered. On the other hand, the average molecular weight per end-group after collagenolysis is only about 500.[16,17] These findings substantiate the multi-stranded structure proposed for collagen in solution on the basis of other types of evidence[9,49-51] and also tell us something about the inter-chain bonding. Though other types of bonding or aggregation cannot be excluded, if the forces involved in holding the digested macromolecule together are largely the residual hydrogen bonds (as the dialysis and optical rotation experiments seem to suggest), a crude estimate of the number of intact bonds needed to hold a fragment to the other strands (at about 10°) can be made. If we assume that the larger peptide fragments (*i.e.*, molecular weight of 1000 or more) remain attached to the degraded macromolecule, it would seem that somewhat less than 10 intact hydrogen bonds are needed (less because collagen contains a high percentage of non-hydrogen-bonding proline). It is interesting, in this connection, that both Thomas[47] and Schumaker, *et al.*,[48] found that their data on the enzymatic degradation of DNA indicated that only a short sequence of intact inter-chain hydrogen bonds (of the order of 3–5) seemed to be needed to prevent the dissociation of the two chains of DNA at room temperature.

TABLE V

RADII OF GYRATION OF VARIOUS MODELS OF BRANCHED RIGID RODS

Model[a]	$(r_g/r_g^0)^b$
a	1.00
b	1.20
c	1.41
d	1.73
e	0.98
f	1.00
g	0.97
h	1.41
i	1.31
j	2.04

[a] Letters correspond to those in Fig. 14. [b] Ratio of radius of gyration of branched model about its center of mass to radius of gyration of unbranched 3-stranded rod (a).

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Appendix

Although the introduction of branches into random coils at constant molecular weight brings about a moderate *decrease* in radius of gyration (r_g),[56] it may be easily seen that creating branches in a rigid rod *without* an accompanying increase in back-bone flexibility generally results in a moderate *increase* in r_g. Fig. 14 presents schematically some representative two-dimensional models of various ways in which a rigid three-stranded structure such as the native ichthyocol "monomer" might branch by the partial "fraying-out" of single strands from the back-bone after enzymatic cleavage. The ratio of the radius of gyration of each model about its center of mass to that of the original three-stranded rod (Fig. 14a) has been determined geometrically, taking the length of each sub-unit rod to be much greater than its diameter. These ratios are presented in Table V. Note that r_g/r_g^0 is larger than unity in most cases, and exceeds 2.0 in the extreme case of Fig. 14j.

In these model calculations it has been assumed that both the "back-bone" and the branches remain rigid after "fraying-out." Clearly the effect of introducing flexibility into the "frayed-out" branches is to reduce r_g/r_g^0 somewhat in each case, by moving the center of mass of the branch in toward the center of mass of the whole structure. By introducing flexibility into the back-bone as well, we approach the branched random coil model discussed in the text.

BETHESDA, MARYLAND
NEW YORK, NEW YORK