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THE DENATURATION AND RENATURATION OF EARTHWORM-CUTICLE COLLAGEN

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SUMMARY

Purified earthworm-cuticle collagen has a denaturation temperature of 23° C. When heated earthworm-cuticle collagen is cooled, many of the denatured particles return to the native structure. Intact earthworm-cuticle collagen is very resistant to trypsin but is degraded by collagenase (EC 3.4.4.19). When earthworm-cuticle collagen is heat-denatured, it splits into three components, analogous to the α, β, and γ components of vertebrate tropocollagen. These earthworm-cuticle collagen components have $s^0_{20,w}$ values expected for peptide chains derived from particles twice as long as vertebrate collagen macromolecules. It is concluded that earthworm-cuticle collagen belongs to the collagen class of proteins. The high hydroxyproline content (153 residues/1000 total residues) and the intermediate transition temperature (23°) substantiates the hypothesis that the steric arrangement of imino acids stabilizes the collagen helix. The possibility that hydrogen-bonding from hydroxyproline contributes further stability is eliminated.

INTRODUCTION

The major protein component of the cuticles of earthworms (probably Lumbricus) and Ascaris has been identified as collagen on the basis of wide angle X-ray diffraction data[1,2] and amino acid composition[3–5]. However, the collagen fibrils in the cuticles do not exhibit the periodic cross striations that have been observed in other collagens examined with the electron microscope[5,6].

A previous report[5] described the isolation, purification, and physical-chemical characterization of the acetic acid-soluble collagen from the cuticles of common night crawlers. In brief summary, the findings of that investigation were: that on the basis of amino acid analysis, the extracted protein was collagen; that the soluble earthworm-cuticle collagen particles are relatively rigid rods having the same diameters as vertebrate tropocollagen particles, but that the EWCC rods are about twice as long and have about twice the molecular weight as do TC rods; that EWCC and TC solutions have about the same optical rotatory powers; that EWCC particles do not precipitate as ordered aggregates when subjected to the same conditions that produce aggregation in TC solutions; and that purified EWCC contains about

Abbreviations: EWCC, earthworm-cuticle collagen; TC, tropocollagen from various vertebrate sources; CSC, calf-skin collagen; Ichthyocol, soluble collagen from carp swim bladder.

10% (by weight) of carbohydrate that is intimately associated with the protein moiety. A comparison of EWCC and TC is shown in Table I.

The reversible collagen ⇄ gelatin transition is a well known phenomenon of several vertebrate collagens and has recently been reviewed by HARRINGTON AND VON HIPPEL[7]. TC particles in solution, when heated or placed in "melting solvents" such as urea or KCNS, undergo marked changes in properties such as viscosity, sedimentation rate, optical rotation, and morphology. These changes occur over a relatively narrow temperature range and are reversed to some extent after the TC particles are cooled or returned to the original solvent[8–11]. These phenomena have been interpreted as manifestations of helix ⇄ random coil or crystalline ⇄ amorphous state transitions[8,12,13]. The transition temperature depends upon the source of the collagen. The transition temperature differs by a constant amount from the temperature at which shrinkage occurs in gross fibrous collagenous tissue from the same source, leading to the conclusion that the transition in dilute solution and the shrinkage are, in effect, the same phenomenon; the shrinkage temperature is higher because of the additional stabilization energy inherent in the solid state[7].

TABLE I

COMPARISON OF EWCC AND TC

	$\bar{v}$ (ml/g)	$s^0_{20,w}$ (S)	$[\eta]$ (dl/g)	$[\alpha]_D$	Diameter (Å)	Length (Å)	$M \times 10^{-5}$
EWCC*	0.70	4–5	28–35	−390°	14–17	5000–5600	6.0–7.0
TC**	0.70	2.8–3.5	11.5–16.5	−350° to −415°	14–17	2400–3500	2.2–3.6

* From MASER AND RICE[5].
** Selected values from HARRINGTON AND VON HIPPEL[7].

Several authors have noted the apparent correlations between the transition and/or shrinkage temperature on the one hand, and the proline, hydroxyproline, or total imino acid content of various collagens on the other, and have explained the relationships on the basis of the additional structural stability presented to the collagen molecule by these entities[8,14–16].

The present communication describes experiments on the thermal denaturation and renaturation of EWCC, and the action of the enzymes collagenase (EC 3.4.4.19) and trypsin (EC 3.4.4.4) on EWCC. The results provide evidence confirming the collagenous nature of EWCC, and further elucidate the structure and properties of this unique collagen. In addition, the data are discussed as to their bearing on the relationship between the chemical composition and the melting temperature of collagens.

METHODS AND RESULTS

General

Acetic acid-soluble EWCC was isolated and purified by the previously described procedures[5] and stored in lyophilized form. For experimental use, the lyophilized EWCC was dissolved in 0.5% acetic acid by gentle homogenization in a hand tissue grinder followed by gentle stirring for 24 h, after which insoluble material and aggre-

gates were removed by centrifugation at 78000 × *g* for 2 h. One preparation was concentrated by pervaporation in the cold room to avoid shearing upon homogenizing lyophilized material. Lyophilized soluble calf-skin collagen and ichthyocol from carp swim bladders were prepared for experimental use in the same manner as was EWCC, and were used as controls. All of the preparative procedures were performed at 0–4°. Transfer of solvent was effected by dialysis of the acetic acid solutions of protein against the appropriate buffers. Protein concentrations were estimated either by micro-Kjeldahl or colorimetric[17] nitrogen analyses, assuming nitrogen contents, by weight, of 14% for EWCC and 17% for CSC and ichthyocol.

Enzyme experiments

The action of trypsin and collagenase on the collagens was monitored by viscometry. Measurements were made with any of four Cannon–Ubbeholde dilution viscometers having flow times ranging from 200 to 600 sec for water at 15°. The viscosities of the collagen solutions were independent of the rate of shear within the range used. The temperature of measurement was controlled by means of a constant-temperature water bath regulated to within 0.05°.

Trypsin and collagenase were obtained from Mann Research Laboratory (lot No. F3892) and Worthington Biochemical Corporation (lot No. 62523), respectively. The enzymes were dissolved in water at a concentration of 0.2% (w/v), cleared by centrifugation, and dialyzed against the same buffers in which the collagens were dissolved. The enzyme solutions were added to the substrates directly in the viscometers by removing with a long pipette about half of the protein solution in the viscometer reservoir, adding the enzyme solution, and replacing the protein solution. In this manner, fast efficient mixing of the enzyme and substrate was obtained, with little loss of enzyme on the walls of the viscometer. In addition, the enzymic action could be studied on the same solutions from which control viscosities had been previously obtained. Usually, 0.01 ml enzyme solution was added to either 2 or 5 ml protein solution. The ratio of enzyme to substrate, by weight, varied from 1:10 to 1:50. The time at which the enzyme solutions were added was recorded as zero time. Flow times of the solutions varied from 4 to 10 min. The time of measurement was recorded as the time halfway between the beginning and end of a measurement.

The buffer used for the collagenase experiments was Tris (pH 6.9), 0.05 M with respect to Tris, and either 0.05 or 0.5 M with respect to $CaCl_2$. It was found that EWCC and CSC were insoluble in the higher calcium concentration buffer used for ichthyocol.

The experimental results are shown in Figs. 1 and 2, which are plots of the viscosities of the substrates *vs.* time after enzyme addition. The ordinate of Fig. 1 is expressed in absolute viscosity units, while that of Fig. 2 shows the same data expressed as fractions of the viscosities at zero time. All measurements were made at temperatures below the transition temperature of the collagen, so that the enzyme acted on native collagen, and not on gelatin. A drop in viscosity is an indication of enzyme activity in which the collagen particles were either broken into shorter segments or were collapsed to some extent from rigid structures. Each of the two enzymes had a characteristic effect on the viscosities of the substrates, regardless of the source of the collagen. Trypsin produced a small initial viscosity drop, after which very little effect was observed. A small fraction of this initial drop may have been due to

the dilution which occurred upon the addition of the enzyme solution to the protein. Trypsin caused the ichthyocol viscosity to drop to about 80% of its initial value, but the EWCC viscosity dropped only to 97% even though the enzyme: substrate ratio was about 7 times higher in the case of the EWCC. This difference may have been due to the different buffers used.

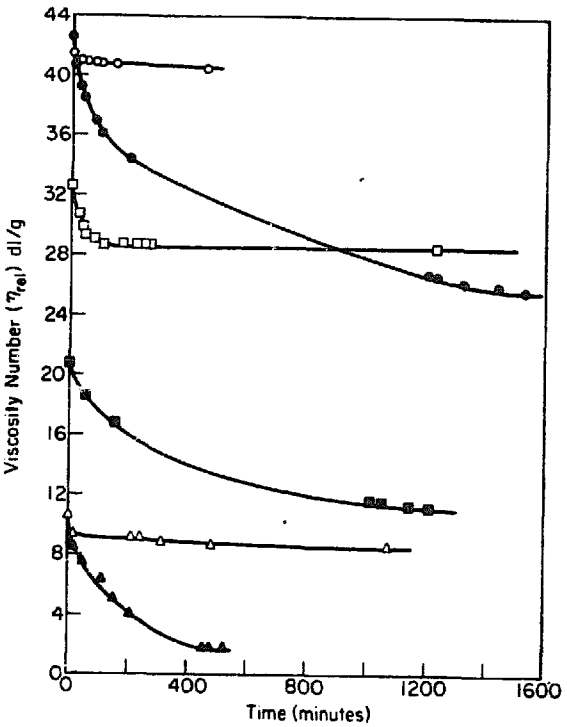

Fig. 1. Viscosities of collagen solutions *vs.* time of enzymic action. O—O, 10 γ tryspin on 61 γ earthworm-cuticle collagen (EWCC), phosphate, 15.5°. ●—●, 20 γ collagenase on 122 γ EWCC, Tris, 0.05 M $CaCl_2$, 14.4°. □—□, 10 γ trypsin on 200 γ calf-skin collagen (CSC), phosphate, 15.5°. ■—■, 20 γ collagenase on 206 γ CSC, Tris, 0.05 M $CaCl_2$, 14.4°. Δ—Δ, 10 γ trypsin on 440 γ ichthyocol, Tris, 0.5 M $CaCl_2$, 17.9°. ▲—▲, 10 γ collagenase on 440 γ ichthyocol, Tris, 0.5 M $CaCl_2$, 17.9°.

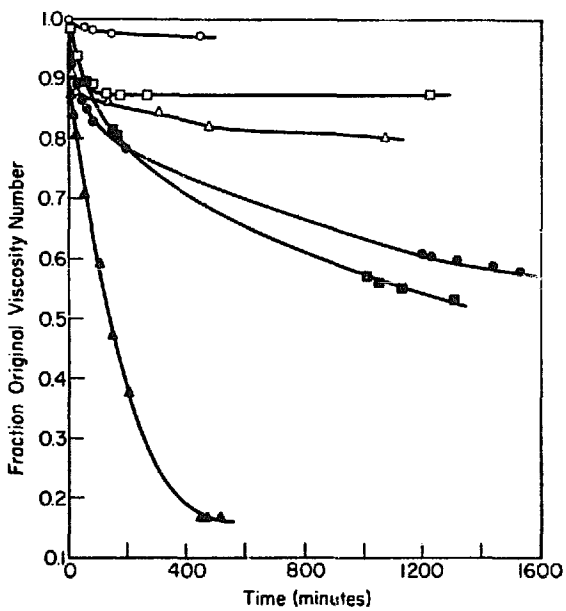

Fig. 2. Fraction of original viscosity *vs.* time of enzymic action. The meaning of the symbols is the same as in Fig. 1.

The trypsin action became extremely slow after a relatively short time, and was obviously in contrast with that of collagenase. The shapes of the collagenase curves are similar for all three substrates. The viscosities all dropped sharply at first, and then more slowly, but constantly, over periods of several hours. The ultimate viscosity values were probably not reached, even though some of the experiments lasted more than 20 h. The action of collagenase on ichthyocol was faster and produced a lower ultimate viscosity, even on a percentage basis, than on EWCC and CSC.

Thermal denaturation and renaturation experiments

Electron microscopy: Collagen solutions were prepared for electron microscopy by a mica-replica technique[18,19] in which micro-droplets of the protein solutions were sprayed onto the surface of freshly cleaved mica, shadowed with platinum at a low angle, and replicated with carbon. This method enables particles with one dimension as small as 15 Å or less to be visualized. The specimens were placed in the vacuum

evaporator within about 15 sec after spraying, and the bell jar was immediately evacuated. An aliquot of 0.005% EWCC in 0.5% acetic acid was sprayed at 4°. The remaining solution was heated at 70° for 15 min, and an aliquot was sprayed at this temperature. The final aliquot was cooled at 4° for 24 h and sprayed at this

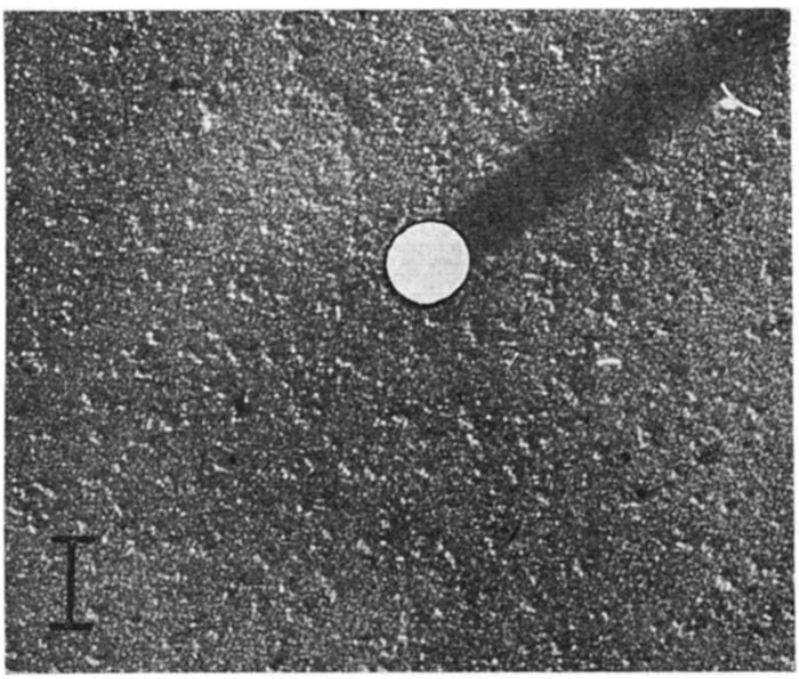

Fig. 3. Electron micrograph of a mica-replica of an 0.005% solution of earthworm-cuticle collagen heated at 70° for 15 min and sprayed in an oven at 70°. No anisometric particles are seen. 97000 ×, magnification line is 1000 Å.

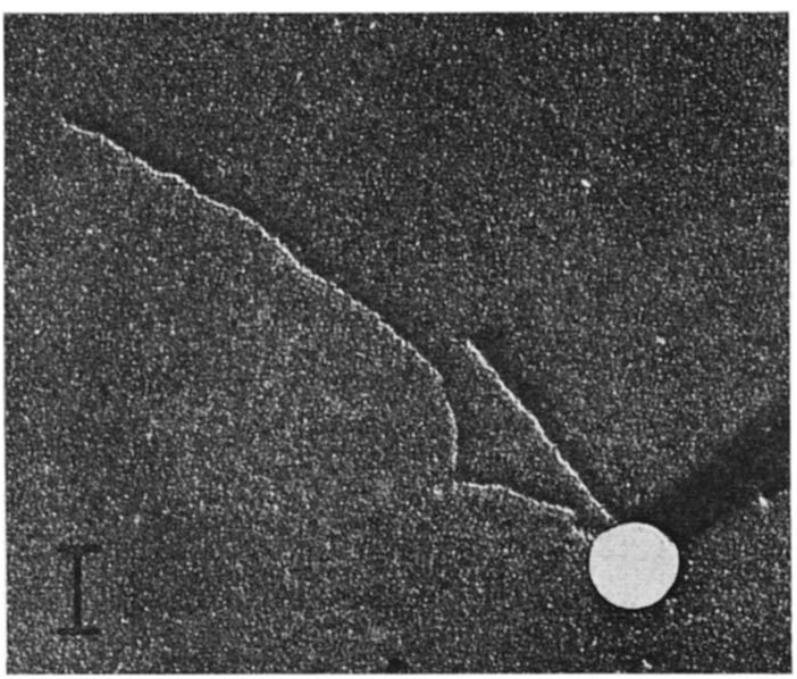

Fig. 4. Electron micrograph of a mica-replica of an 0.005% solution of earthworm-cuticle collagen heated at 70° for 15 min, cooled at 4° for 24 h and sprayed at 4°. Long, narrow particles are visible. 96000 ×.

temperature. Figs. 3 and 4 show micrographs of the heated and of the heated and cooled preparations respectively. (Fig. 2 of ref. 5 showed an example of untreated EWCC.) Before heating the particles are long, relatively rigid rods with diameters of about 15 Å. The particles, when sprayed while hot, have no obvious asymmetry, as is seen in Fig. 3. No long rods were observed on any of 15 microscope grids prepared from three different sprayings of hot EWCC. Upon cooling, some of the EWCC particles recover their original morphology (Fig. 4) and are indistinguishable from the particles photographed before heating.

Viscosity and optical rotation: Viscosity measurements were made using the same viscometers and methods described for the enzyme experiments. Optical rotation measurements were made with a Schmidt and Haensch Polarimeter, employing sodium D line illumination. Solutions were measured in a water-jacketed cell having a length of 1 dm and a volume of 2.8 ml. The temperature of the cell was controlled

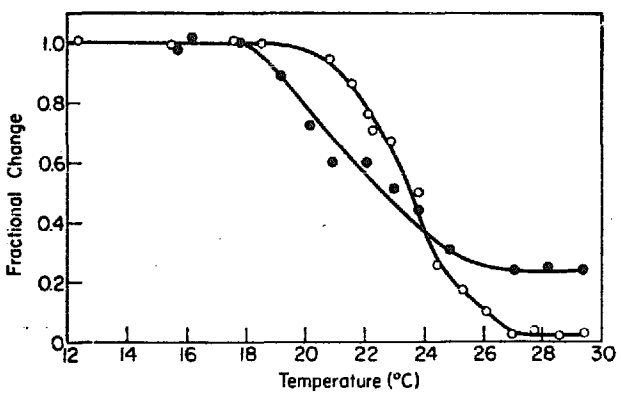

Fig. 5. Fractional change of viscosity (O—O) and optical rotation (●—●) upon heating solutions of earthworm-cuticle collagen (EWCC). Phosphate buffer. Original viscosity of the 0.005% EWCC solution was 31.5 dl/g; final viscosity 0.9 dl/g. Original $[\alpha]_D$ of the 0.1% EWCC solution was −375°; final $[\alpha]_D$ −92°. Denaturation temperature (T_d) is 23.5° for viscosity and 22.7° for optical rotation.

by water circulating from a constant-temperature reservoir regulated to within 0.05°. No temperature difference was observed between the cell and the reservoir. Solutions were heated directly in the viscometer or polarimeter cell at rates of from 2° to 4°/h, and measurements were made as the heating progressed. The temperatures recorded are the means of the temperatures at the beginning and end of the measurements. The rate of heating, within the range used, had no observable effect on the results. The buffer solvent used for both viscosity and optical rotation was phosphate (pH 7.4, I 0.4). The concentrations of EWCC used were 0.005% for viscosity and 0.1% for optical rotation. After the EWCC solutions were heated to the maximum temperatures required by the experiments, they were stored at 4°, and were measured periodically thereafter at 10°.

The results of one heating experiment are shown in Fig. 5. The viscosity dropped from an initial value of 31.5 dl/g to less than 1 dl/g. The levorotation dropped from −375° to −92°. The transition temperature (T_d), defined as the temperature at which the measured quantity is half its original value[20], was 22.7° for optical rotation and 23.5° for viscosity. It was consistently observed that the optical rotation began changing and approached its ultimate value at a slightly lower temperature than did the viscosity. After cooling, the optical rotation returned to an apparent limiting

value of −248° within 41 h; the viscosity returned to a value of only 2.8 dl/g after 11 days of cooling.

Sedimentation velocity: Measurements were made with a Spinco Model-E ultracentrifuge equipped with schlieren optics. EWCC solutions, at concentrations of 1.0, 0.7 and 0.4 % in acetate buffer (pH 4.8, I 0.15) were centrifuged in a Spinco An-C-F 4-hole rotor at 52640 rev./min. Aliquots of the three EWCC solutions were centrifuged at 10°. The remaining solutions were heated for 15 min at 70°, placed in preheated cells in a preheated rotor, and centrifuged at 31.6°. Aliquots of the solutions, after heating, were stored at 4° for 48 h and then centrifuged at 10°. Schlieren patterns from one such experiment are shown in Fig. 6. The single hypersharp peak shown by unheated EWCC (Fig. 6A) is typical of the appearance of all types of intact collagen in the ultracentrifuge. The EWCC splits into two and sometimes three peaks when heated. An example of three peaks obtained in this experiment is shown in Fig. 6B.

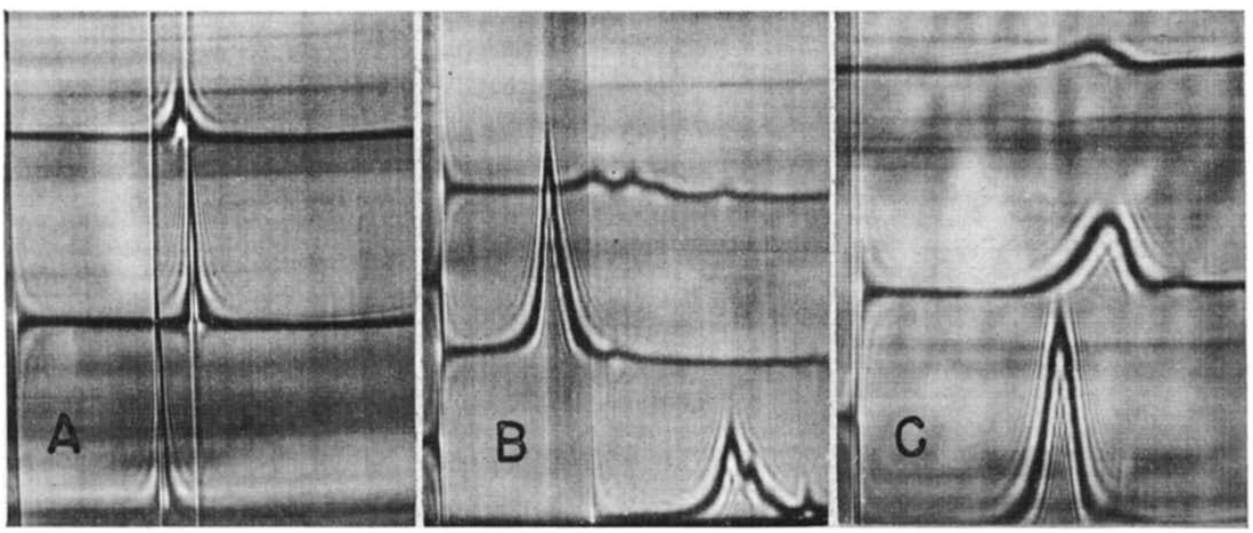

Fig. 6. Velocity sedimentation schlieren patterns of 1 %, 0.7 % and 0.4 % earthworm-cuticle collagen (EWCC) in sodium acetate buffer (pH 4.8, I 0.15) in three cells of a Spinco An-C-F 4-hole rotor. Sedimentation is from left to right. (A) unheated EWCC after 200 min at 52640 rev./min, 10°, phase plate angle 60°; (B) EWCC heated 15 min at 70° after 48 min at 52640 rev./min, 31.6°, phase plate angle 65°; (C) EWCC heated 15 min at 70° and cooled at 4° for 48 h, after 181 min at 52640 rev./min, 10°, phase plate angle 55°.

This behavior is comparable to that exhibited by vertebrate TC as has been noted by several investigators[10,11,21–24]. The three components in TC have been designated α, β, and γ, with α the slowest moving peak and γ the fastest moving. The three EWCC peaks are similar in relative abundance and relative rate of sedimentation, although not in absolute sedimentation rates, to those of TC, and we have similarly designated them α, β, and γ. The relative amounts of the components varied from preparation to preparation. The large γ component shown in Fig. 6B represents the highest concentration we have observed for this component. When the heated solution was cooled, only one major peak similar to that of unheated EWCC was observed; a small faster peak is also visible in Fig. 6C.

Fig. 7 shows plots of the reciprocals of the corrected sedimentation coefficients of the components shown in Fig. 6 *vs.* protein concentration. Values from a duplicate experiment at the higher concentration are also included for the experiment at 31.6°.

The mean $s^0_{20,w}$ for five preparations of undenatured EWCC was 4.50 S (see ref. 5). The extrapolation for unheated EWCC in Fig. 7 (I) gives $s^0_{20,w} = 4.08$ S. Renatured EWCC extrapolated to $s^0_{20,w} = 4.75$ S in Fig. 7 (II). Extrapolations for the three components of heated EWCC are also shown in Fig. 7 (III). α, $s^0_{20,w} = 6.25$ S; β, $s^0_{20,w} = 8.85$ S; γ, $s^0_{20,w} = 13.3$ S.

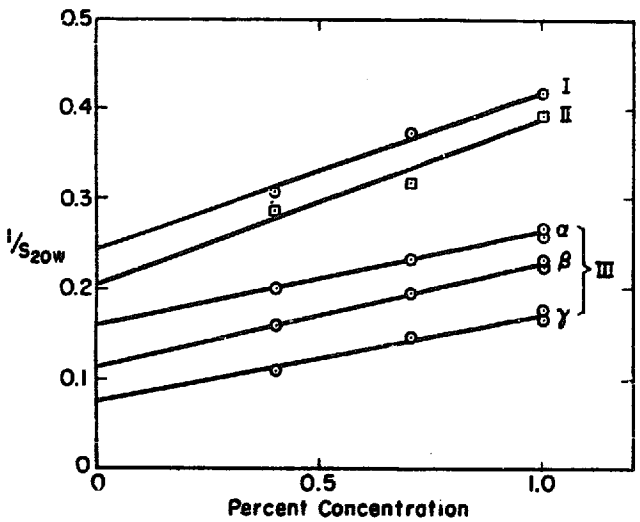

Fig. 7. Extrapolations of $1/s_{20,w}$ *vs.* concentration of earthworm-cuticle collagen (EWCC) solutions shown in Fig. 6 and described in text. I, unheated; $s^0_{20,w} = 4.08$ S; II, heated and cooled, $s^0_{20,w} = 4.75$ S; III, heated at 70°, 15 min and centrifuged at 31.6°. α, $s^0_{20,w} = 6.25$ S; β, $s^0_{20,w} = 8.85$ S; γ, $s^0_{20,w} = 13.3$ S.

DISCUSSION

Additional evidence for the collagenous nature of EWCC

Although EWCC has been shown to have several characteristics unique to the collagen class of proteins[3–5], the several distinct differences[5] between EWCC and vertebrate collagen suggested the gathering of further evidence that EWCC is, in fact, collagen. The most significant differences are that the gross fibrils from which EWCC was extracted are not striated, that EWCC particles are about twice as long as TC particles, that intact EWCC cannot be aggregated into the specific "fingerprint" particles characteristic of TC, and that EWCC contains a relatively large amount of strongly attached carbohydrate.

In all of the experiments described in this communication, EWCC behaved as would be expected of a collagen, the particles of which were twice as long as those of previously described collagens. The effects of heating and cooling vertebrate TC have been investigated in the past by electron microscopy, viscosity, optical rotation, and sedimentation velocity, and have been shown to be similar to the effects on EWCC as related herein. These results will be more fully discussed subsequently.

The action of collagenase and trypsin is very significant in establishing EWCC as collagen. Although trypsin has been used to hydrolyze heat-denatured or gelatinized collagens into many oligopeptides[7,25,26], it has been shown that undenatured collagen is only slightly sensitive to tryptic action at low temperatures[27]. The trypsin curves shown in Figs. 1 and 2 are in good agreement with those published by these authors. The unusual resistance of collagen to trypsin is probably a manifestation of the unique

chemical structure of collagen, and the similarity between the data for EWCC and that of CSC and ichthyocol indicates that EWCC possesses the structural properties responsible for the resistance of trypsin. The relatively higher resistance of EWCC to trypsin may be explained by the suggestion that EWCC has fewer chains extending from its helical portion than do other collagens. Chains extending from the helical portion of TC were first postulated by BOEDTKER AND DOTY[20] and later invoked by HODGE *et al.*[27] to explain tryptic digestion of TC.

Collagenase, extracted from culture media of *Clostridium histolyticum* has never been found to attack any protein other than collagen or gelatin, a property probably related to the unique amino acid content of collagen and the unusual polypeptide chain structure resulting therefrom[28,29]. Figs. 1 and 2 show that the effect of collagenase on the viscosity of EWCC solutions is similar to its effect on the viscosities of CSC and ichthyocol. The viscosities of the collagen solutions dropped to much lower values than when they were attacked by trypsin, and the data indicate that, at least in the cases of CSC and EWCC, the lower limiting values of the viscosities had not been reached at the conclusion of the experiments. Several investigators have reported similar experiments performed on vertebrate TC solutions, and their results are in agreement with those reported here[27,30].

The relatively slower viscosity drop for both EWCC and CSC as compared to ichthyocol may be a result of the difference in calcium concentration. Calcium is apparently an activator of collagenase[28]. However, the very high ratio of hydroxyproline to proline in EWCC should be noted. Collagenase is specific for the sequence –Pro·X·Gly·Pro·Y·– and substitution of both proline residues by hydroxyproline depresses enzymic activity in synthetic substrates[31]. It is likely that most of these sequences in EWCC are –Hypro·X·Gly·Hypro·Y·– since EWCC contains 153 Hypro and only 11 Pro per 1000 total amino acid residues[5].

We believe that the manner of action of trypsin and collagenase on EWCC, when combined with the amino acid composition of EWCC and the effects of heating and cooling on EWCC solutions provides strong evidence that EWCC is one of the collagen class of proteins.

Preliminary wide-angle X-ray diffraction photographs of purified EWCC pulled into fibers gives further evidence of the collagen character of this protein. We have obtained strong 2.86-Å reflections and rather weak 10–11-Å arcs.

Thermal denaturation and renaturation

HARRINGTON AND VON HIPPEL[7] have summarized the evidence that collagen undergoes a helix $\rightleftharpoons$ random coil transition. Apart from the kinetic studies of the transition, the bulk of this evidence has been derived from experiments on vertebrate TC similar to those described in this report on EWCC.

The direct visual observation of the EWCC particles by electron microscopy shows that the particles lose their asymmetry at elevated temperatures, but that the asymmetry returns to a great degree upon cooling the particles. RICE[9] interpreted the results of similar experiments on a variety of collagens to be due to the collapse and reformation of the helical structure of some of the triple polypeptide chains composing the particles. The small globules seen in Fig. 3 may be the collapsed particles.

Optical-rotation and viscosity experiments on heated and cooled TC solutions have provided very strong evidence for the transition[8,33,34]. Again, our results for

these measurements on EWCC agree with those observed for TC, in that the transition occurred over a relatively narrow range of temperature, that the transition as measured by both methods was similar, and that the return after heating to helical structure was evidenced by a fast recovery in the optical rotation (41 h), but a very slow recovery in the case of the viscosity (several weeks). The rise in levorotation has been interpreted as being due to the reformation of the helical structure of the individual polypeptide chains, while the viscosity rise is due to the much slower super coiling among the polypeptide chains[7]. The slightly faster drop of optical rotation as compared to viscosity (Fig. 5) may indicate that EWCC and TC do have slightly different structures. Similar curves for CSC can be superimposed[8].

The observation of three components of hot EWCC in the ultracentrifuge is another similarity between EWCC and TC. The α, β, and γ peaks of the latter have been interpreted as one, two, and three polypeptide-chain components, respectively[8,10,11,21,22]. The chains of the heavier components are thought to be held together by covalent cross links, rather than by weaker bonds that would break with an increase in temperature. The sedimentation constants of the EWCC α, β, and γ peaks are higher than are those of vertebrate TC. This is to be expected if the EWCC polypeptide chains are about twice as long as those composing TC particles. The variability of the amounts of the three components in heated EWCC can be attributed to the large number of individual worms required for a single preparation. It is known that young tissue yields less γ component than older, more highly cross-linked collagen. The higher $s^0_{20,w}$ for EWCC α, β, and γ components shows that strong bonds (*i.e.*, covalent) hold together the intact "dimer" of EWCC even during thermal denaturation.

To date, all attempts to produce ordered aggregates from intact EWCC have failed, but typical segment long spacing has been formed from EWCC subjected to shearing forces. The sheared particles behave as if they were of the same size and shape as undenatured TC. This cleavage is distinctly different from that described above for heat-denatured EWCC. The results of shearing EWCC will be the subject of a future publication.

It will be recalled that the transition temperature (T_d) of EWCC is about 23°. As was stated in the introduction, correlations have been made by several investigators between T_d and proline, hydroxyproline, and total imino acid content of various collagens. EWCC has the highest hydroxyproline content (153 residues/1000 total residues) and the lowest proline content (11 residues/1000 total residues) of any collagen yet reported[4,5]; however, the T_d of EWCC is intermediate among those of the other collagens. HARRINGTON AND VON HIPPEL[7] have tabulated the T_d and imino acid compositions for several collagens. This tabulation is presented in the form of scatter diagrams in Fig. 8, into which our values for EWCC have been introduced. Although the correlation coefficient (r) is not accurate when calculated for small numbers of data such as are presented here, the differences we wish to point out are large enough so that the use of r in an approximate sense may be justified. If the EWCC data are not included, r approaches 1.0 for all three of the scatter diagrams in Fig. 8. If the EWCC data are included, r is less than 0.5 for the plots of proline and hydroxyproline content, but r remains close to 1.0 for the total imino acid content plot. The EWCC points are so far removed from the regression lines in the first two cases that they destroy to a large extent the correlations which were statistically,

at least, as valid as that for the total imino acid content. The correlation in the third case, however, remains as good as before when the earthworm data are included.

These results indicate that the structural stability of collagen is related to the total imino acid content, rather than to either of the two imino acid contents, individually. GUSTAVSON[14] attributed the apparent relationship between hydroxyproline content and thermal stability to the stabilizing force of interchain hydrogen bonds between the hydroxyl groups of hydroxyproline and proximal oxygens of carbonyl groups. Proline, on the other hand, is not a hydrogen donor and cannot participate in hydrogen bond formation as can hydroxyproline. On the basis of the imino acid content and T_d of EWCC, then, it would appear that structural stability to heat in collagen rests on some features other than hydrogen-bonding.

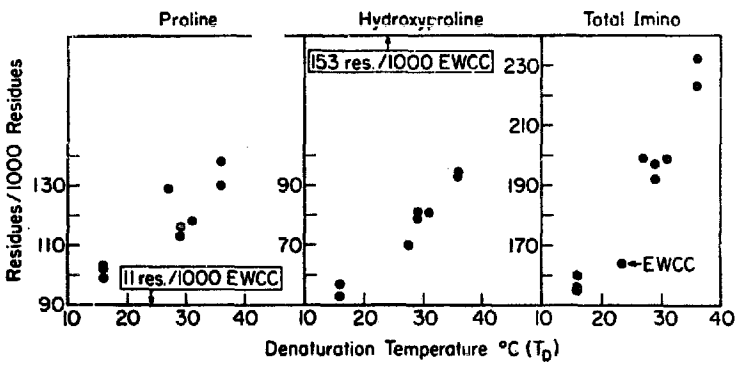

Fig. 8. Scatter diagrams of proline, hydroxyproline, and total imino acid content in terms of residues per thousand total residues *vs.* denaturation temperature. Data derived from HARRINGTON AND VON HIPPEL[7] tabulation for a variety of collagens. Arrows indicate our data for earthworm-cuticle collagen (EWCC). Note that data for EWCC correlates only with scatter diagram for total imino content.

Evidence does exist from studies of the synthetic polymer poly-L-proline that structural stability can be imparted to polypeptide chains from the steric properties of proline or hydroxyproline alone, either by the configurational energy of these residues, or by hydrogen bonds among water molecules which are closely associated with the polypeptide chains as a result of the particular configuration of the imino acid residues[35]. The data reported here for EWCC support the latter hypothesis. Stabilization by hydrogen bonds is not consistent with the results of our experiments.

The large carbohydrate content (10–13 %) of purified EWCC might be expected to interfere with the various hydrodynamic and enzymatic studies reported here. However, the agreement of data obtained with EWCC and with CSC and ichthyocol indicates no obvious participation of the carbohydrate moiety in either helix $\rightleftarrows$ coil transformations or enzymatic susceptibility. Preliminary attempts to remove carbohydrate from intact EWCC by trypsin, lysozyme, and pectinase have failed. Exhaustive digestions of denatured EWCC with trypsin, papain, and collagenase indicate that the carbohydrate is bound to peptides by bonds not cleaved by these enzymes.

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