SOLUBLE EARTHWORM CUTICLE COLLAGEN: A POSSIBLE DIMER OF TROPOCOLLAGEN

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ABSTRACT

When soluble earthworm cuticle collagen molecules are subjected to the shearing forces of a flow birefringence instrument, they are broken into particles approximately half the original size. The broken particles resemble vertebrate tropocollagen molecules in their hydrodynamic properties, in levorotatory powers, and in their appearance in the electron microscope. Most significantly, the broken earthworm particles form ordered aggregates similar to the segmented-long-spacing aggregations formed by vertebrate tropocollagen. These phenomena are explained by the suggestion that earthworm collagen molecules are dimers of tropocollagen-like particles. On this basis, an explanation is presented for the lack of striations in the gross collagen fibrils of earthworm cuticle.

INTRODUCTION

We have described, in two previous reports (1, 2), the physical-chemical characterization, the effects of certain enzymes, and the thermal denaturation and renaturation behavior of the acetic acid–soluble collagen from the cuticles of common night crawler earthworms (probably *Lumbricus*). This collagen is of particular interest because the gross collagen fibrils in the cuticle do not exhibit, in the electron microscope, the periodic transverse striations that are commonly observed in other collagens (1, 3). In spite of the lack of striations, there is substantial evidence that soluble earthworm cuticle collagen (EWCC) belongs to the collagen class of proteins. This evidence is based primarily on wide angle x-ray diffraction (4, 5), amino acid analyses (1, 6, 7), optical rotation (1), the action of collagenase and trypsin (2), and thermal transition (2) studies. However, soluble EWCC has several unique properties that warrant further investigation.

The most pertinent differences between EWCC and vertebrate tropocollagen (TC) are: that EWCC appears, from hydrodynamic and electron microscopical investigations, to be about twice as long as TC and to have about twice its molecular weight; that EWCC contains about 10 per cent by weight of closely associated carbohydrate, although TC is almost completely free from carbohydrate; and that intact EWCC cannot be precipitated by the usual methods into the highly ordered aggregates that are characteristic of TC (1). These ordered aggregates are formed by the highly specific alignment of TC particles when they are subjected to particular environmental conditions of pH and ionic character (8).

This communication describes investigations of the structure of EWCC relevant to its unique properties.

METHODS AND RESULTS

General

Acetic acid–soluble EWCC was isolated and purified by the previously described procedures

(1) and stored in lyophilized form. For experimental use, the lyophilized EWCC was usually dissolved in 0.5 per cent acetic acid by gentle homogenization in a hand tissue grinder, followed by gentle stirring for 24 hours. Insoluble material and aggregates were removed by centrifugation at 78,000 *g* for 2 hours. Change of solvent was effected by dialysis of the acetic acid solutions of EWCC against the appropriate buffers. The EWCC concentrations were determined by either micro-Kjeldahl or Johnson nitrogen analyses, assuming a nitrogen content for EWCC of 14 per cent by weight (1, 6, 7).

Streaming Birefringence

Streaming birefringence measurements were made with a Rao Flow Birefringence viscometer using a 0.5-mm annular gap. The temperature of measurement was controlled by water circulating from a constant temperature bath regulated to within 0.05°C. No difference was observed between the temperature of the reservoir and that of the effluent side of the flow birefringence cell. The speed of rotation of the cell was measured with an electronic counter activated by a photocell trained on a black spot on the rotating cell. Measurements were made of extinction angle at

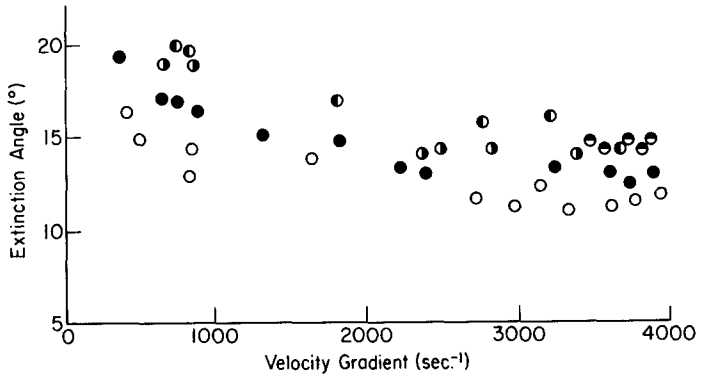

FIGURE 1 ○, 1.24 mg/ml; ●, 0.62 mg/ml; ◑, 0.41 mg/ml; ◐, 0.31 mg/ml; ◓, 0.16 mg/ml. Plot of extinction angle *vs.* velocity gradient for flow birefringence measurements of EWCC solutions in phosphate buffer. The parameters are not dependent above about 2000 velocity gradient, indicating that the particles are not being deformed by the shearing forces. Concentration appears to have no effect below 0.41 mg/ml. Temperature was 14°C.

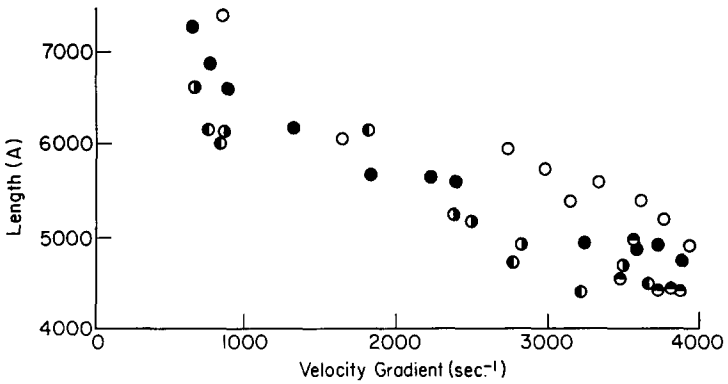

FIGURE 2 ○, 124. mg/m;l ●, 0.62 mg/ml; ◑, 0.41 mg/ml; ◐, 0.31 mg/ml; ◓, 0.16 mg/ml. Plot of calculated length *vs.* velocity gradient for flow birefringence measurements of EWCC in phosphate buffers. The lengths were calculated from the extinction angles plotted in Fig. 1. The lengths in the area in which they are independent of concentration and velocity gradient are about 4500 A. Temperature was 14°C.

several velocity gradient values for several concentrations of two preparations of EWCC. At very low velocity gradients, the extinction angles could not be accurately determined. The solvent was our standard phosphate buffer, pH 7.4, ionic strength 0.4 (2).

The results of these experiments for one of the EWCC preparations are shown in Figs. 1 and 2. Fig. 1 is a plot of extinction angle *vs.* velocity gradient. The independence of these two variables at high velocity gradients indicates that the particles are still rod-shaped even at higher gradients. Fig. 2 is a plot of the calculated lengths

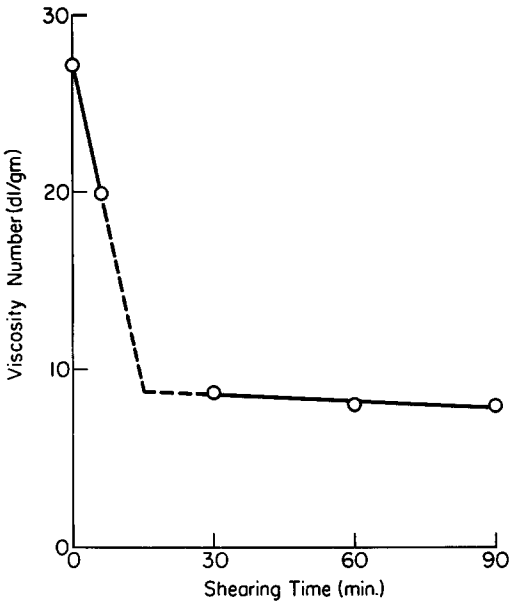

FIGURE 3 Plot of viscosity number *vs.* time of shearing in the flow birefringence apparatus under a velocity gradient of 3000 velocity gradient, for a 0.005 per cent EWCC solution in phosphate buffer. The viscosity number dropped rapidly to an apparent limiting value of about 8 dl/gm, indicating that denaturation was not occurring (cf. Fig. 5 of reference 2). Temperature was 14°C.

of the particles for the same preparation *vs.* velocity gradient. The lengths were calculated on the basis of the assumptions that the particles are relatively rigid and are highly asymmetric. Evidence for these assumptions has been presented in a previous report (1). The calculated lengths for the two EWCC preparations at speeds at which they are independent of the velocity gradient are 4450 A and 3700 A. (A discussion of flow birefringence measurements pertaining to these experiments is given by Leray (9).)

These lengths are considerably less than those calculated from other hydrodynamic experiments, none of which indicated lengths less than about 5000 A (1). We postulated from these considerations that the EWCC particles may have been broken in some manner during the streaming birefringence experiments. To test this hypothesis, we performed several experiments on EWCC solutions after they had been subjected to the shearing forces of the flow birefringence instrument. Except where noted differently, the EWCC

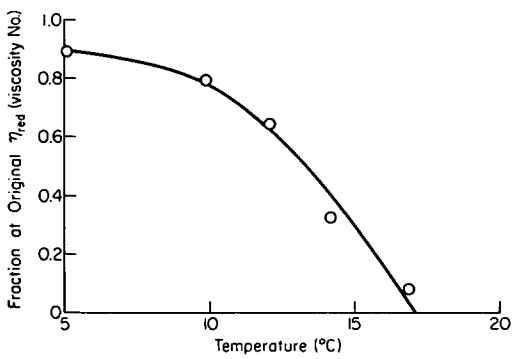

FIGURE 4 Fractions of initial viscosity numbers *vs.* temperature of flow birefringence rotor. Shearing time was 1 hour at each temperature. Viscosities were measured at temperatures of shearing.

solutions used in these experiments were sheared for 1 hour at a velocity gradient of about 3000 sec^{-1}. In experiments in which aliquots of EWCC solutions were removed during the shearing process, the instrument was stopped to permit removal of the sample, and this stopped time was not included in the total time of shearing. Attempts were made to shear EWCC by repeatedly squirting solutions through a no. 27 hypodermic needle at various temperatures. The viscosities of resulting solutions were essentially unchanged, indicating no appreciable change in the particle conformation.

Viscometry

Viscosity measurements were made with Cannon-Ubbeholde dilution viscometers in the manner previously reported (1). Dilute solutions of EWCC in phosphate buffer were sheared at various temperatures. Aliquots were removed

during the shearing and their viscosity was measured at the same temperature at which they were sheared. Fig. 3 is a plot of viscosity number *vs.* time of shearing for one experiment at 14°C. There was an initial sharp drop in the viscosity number, after which it decreased more slowly and reached an approximately limiting value of about 8 dl/gm. In other experiments at this temperature, the final value varied from 8 dl/gm to 11 dl/gm. The magnitude of the viscosity drop, however, was dependent upon the temperature at which shearing occurred. Fig. 4 shows the viscosity numbers, expressed as fractions of the initial viscosity number, of EWCC solutions after 1 hour of shearing *vs.* temperature. The initial viscosity numbers of these preparations varied from 28 to 35 dl/gm. The following experiments were performed on EWCC solutions which had been sheared at 14°C.

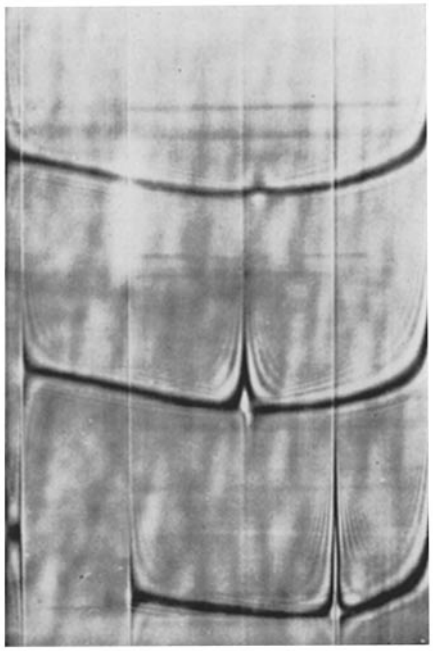

FIGURE 5 Sedimentation velocity pattern of sheared EWCC solutions in phosphate buffer after 198 minutes at 52,640 RPM in a four-hole rotor. Phase plate angle 50°, temperature 10°C. Sedimentation is left to right.

Sedimentation Velocity

Sheared EWCC in phosphate buffer was centrifuged at 52,640 RPM at 14°C in a Spinco model E ultracentrifuge equipped with schlieren optics. The sedimentation coefficients of three concentrations of EWCC were determined and extrapolated to zero concentration. The sedimentation constant, corrected to water at 20°C, was 3.65 S. Fig. 5 shows a schlieren pattern taken from this experiment. The hypersharp peak, typical of collagen, is evident.

Optical Rotation

Optical rotation measurements were made with a Schmidt & Haensch polarimeter, employing sodium D line illumination. A water-jacketed cell 1 dm long, of 7 ml capacity, was used, in which the temperature was controlled to within 0.05°C. Measurements were made at 5.1°C of two sheared EWCC preparations in phosphate buffer at protein concentrations of 0.061 per cent and 0.122 per cent. The optical rotation of the two solutions was −328° and −365°, respectively.

Electron Microscopy

Solutions of sheared EWCC which had been dialyzed against 0.5 per cent acetic acid after shearing were prepared for electron microscopy by the mica replica technique previously described (1, 2). A micrograph of such a preparation is shown in Fig. 6. The collagen particles appear as relatively rigid rods, even after shearing. However, the particles are generally shorter than the typical intact EWCC particles shown previously (1, 2). Several of the particles in Fig. 6 are about 3000 A in length, and in this respect they resemble vertebrate TC particles more than EWCC particles.

Reconstitution Experiments

It will be recalled that one of the primary differences between intact EWCC and TC is that EWCC does not form ordered aggregates. However, when ATP was added to solutions of sheared EWCC in 0.5 per cent acetic acid, a small amount of white precipitate formed. Drops of suspension containing the precipitate were partially dried on carbon-coated microscope grids. The excess was blotted off and the preparation was stained with phosphotungstic acid. A micrograph of such a preparation is shown in Fig. 7. The particles show many fine striations, similar to those seen in the segmented-long-spacing (SLS) form of TC produced by the same method. Some of the fine structure of the EWCC aggregates is obscured by

FIGURE 6 Electron micrograph of sheared EWCC macromolecules prepared by the mica replica technique. × 100,000.

the fibrous background, with which they have always been observed to be in intimate contact. Well defined segments are found scattered throughout the preparations. In Fig. 8 several of the EWCC aggregates are compared with two typical SLS particles formed from calf skin collagen (upper right). The general features of the banding are identical in the two forms. Although the

yields of SLS precipitate were very low (<1 per cent of the total protein), typical segments were observed from three different preparations of sheared EWCC. None of the original EWCC solutions formed a precipitate with ATP before shearing.

DISCUSSION

It is apparent from these results that some of the intact EWCC particles were broken or sheared by the forces in the flow birefringence instrument. It has been previously shown that EWCC has a low thermal transition temperature (24°C), that is, the temperature at which the particles undergo a helix ⇄ random coil or crystalline ⇄ amorphous state transformation (2). This transition begins to occur at less than 20°C. Now the temperature of the solution being sheared in the flow birefringence viscometer, although rigidly controlled on the whole, is probably liable to sharp fluctuations in small local areas. This is due to the restricted area in which shearing occurs (a 0.5-mm annulus), and such changes cannot easily be monitored. So it is likely that at shearing temperatures approaching 20°C, portions of the solutions are, from time to time, subjected to temperatures in the transition range, and the particles in these areas will be denatured from the crystalline state. In a completely denatured EWCC solution, the viscosity number drops to less than 1 dl/gm, or about 1 or 2 per cent of its original value. The point at 16.9°C in Fig. 4 is probably indicative of an approach to the denatured state.

On the other hand, if the particles are to be broken, they must be broken by the destruction of covalent bonds, rather than hydrogen bonds, since thermal denaturation alone does not break the particles into shorter lengths (2). The shearing forces in the flow birefringence instrument cannot be accurately determined, but it can be calculated that the forces used were of the order of magnitude necessary to break covalent bonds; therefore, in that energy range, such breaking would probably be very sensitive to temperature, as a matter completely divorced from that of thermal denaturation. Fig. 4 is an indication of such temperature dependence, and we suggest that at temperatures below about 15°C, little or no denaturation occurs,

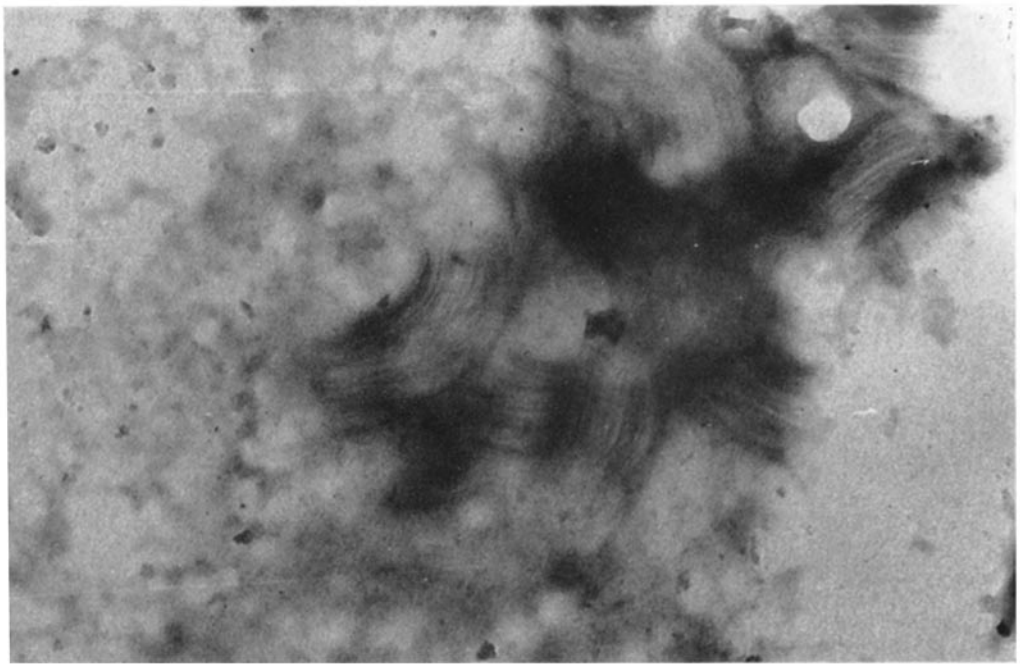

FIGURE 7 Electron micrograph of precipitate of sheared EWCC and ATP showing SLS type aggregates. Stained with 1 per cent PTA in 0.1 M sodium acetate at pH 4.5. SLS from sheared EWCC has always been found associated with masses of unordered fibrous material seen in the background. × 56,500.

but rather that the changes are due to the physical shearing of the EWCC particles.

We were desirous of producing the greatest possible number of sheared particles without subjecting them to denaturing temperatures. For this reason, 14°C was chosen as the shearing temperature. At 14°C, the limiting value of the viscosity number is 7–11 dl/gm, which indicates that little denaturation had occurred. Furthermore, the optical rotation of the sheared material was −328° and −365°, which agrees well with the −390° measured for intact EWCC (2). In heat-denatured EWCC solutions, the levorotation falls to about −100°. The shape of the sedimentation velocity pattern (Fig. 5) is evidence that the sheared EWCC is relatively homogeneous. The electron micrograph in Fig. 6 shows that the sheared EWCC particles are still highly asymmetric, and that they have not been collapsed into the globules produced by denaturation (2).

Assuming then that the EWCC particles have been sheared, and not denatured, the question remains as to the nature of the sheared particles. We interpret the experiments described here as suggesting that the sheared particles are very similar to vertebrate TC particles, and that intact EWCC particles are dimers of TC, or at least contain a TC-like particle as one of their halves.

The sedimentation constant of sheared EWCC is 3.65 S. That of intact EWCC is about 4.5 S, and that of TC is about 3.0 S, although higher values have been reported (10). The viscosity number of sheared EWCC is 7–11 dl/gm as compared with 28–35 dl/gm for intact EWCC and 10–16 dl/gm for TC (2, 10).

The most reliable evidence that sheared EWCC

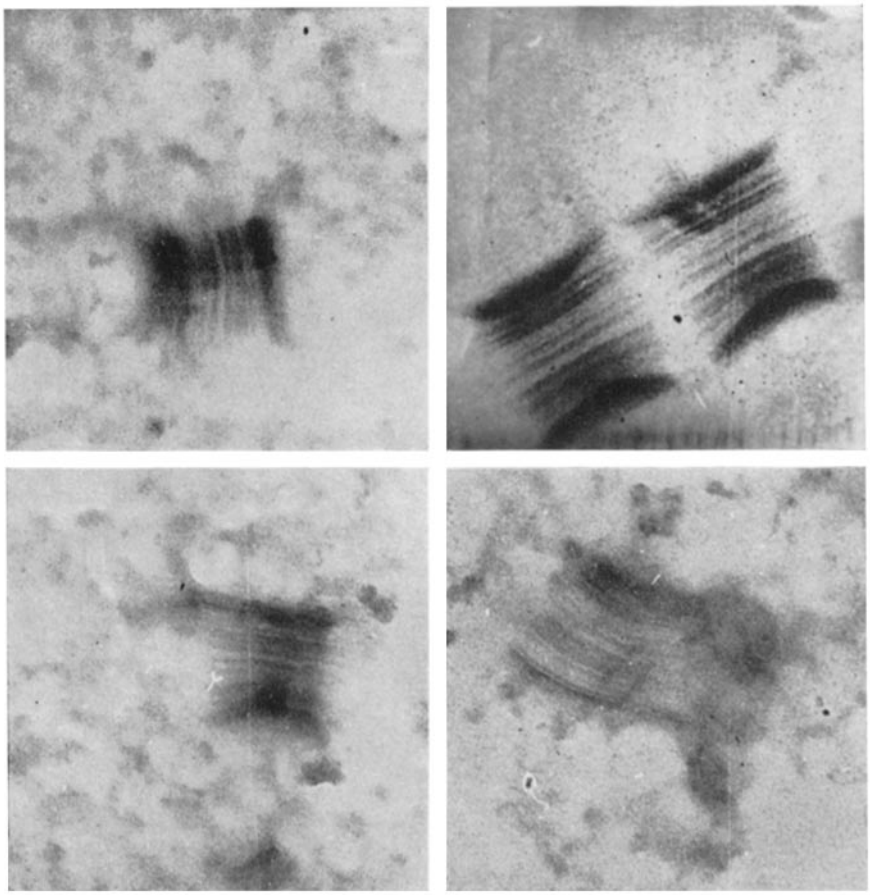

FIGURE 8 Composite electron micrograph of selected SLS particles from sheared EWCC compared with typical SLS from calf skin tropocollagen (upper right). × 74,000.

resembles TC is in the electron micrographs of Figs. 7 and 8. In the SLS form of collagen all the molecules are aligned in the same direction, side by side, so that similar chemical structures are aligned laterally across the molecular aggregate. This alignment produces the detailed banding characteristic of these particles. The SLS particles from EWCC and TC are of approximately the same size, and the major banding features are the same. The formation of SLS has become perhaps the most important criterion for the intactness of collagen molecules (8, 11–14).

The demonstration of SLS particles in precipitates produced by ATP is in itself sufficient evidence to conclude that EWCC macromolecules contain regions virtually identical with vertebrate collagen particles (TC). The contamination of EWCC solutions with TC macromolecules from other sources cannot be completely eliminated, and the relatively low yields of precipitate have suggested this possibility. However, the intact EWCC solutions (which when sheared gave SLS) did not precipitate under the same conditions or with much larger amounts of ATP. We believe that it is unlikely such contamination occurred in three different preparations, and we attribute the low yields to the lack of precise shearing methods available.

It appears, then, that intact EWCC contains portions that are very similar to TC. Since it has been shown that EWCC has about twice the molecular weight and twice the length of TC, it is tempting to postulate that EWCC is a dimer of TC. However, the possibility is not excluded that only a portion of the EWCC particle is TC, that is, forms SLS aggregates, and that the other part is not of the same species. Yet there is limited evidence that supports the dimer hypothesis: the sheared EWCC behaved in the ultracentrifuge as a homogeneous substance; and the optical rotation of sheared EWCC was close to that of intact EWCC and TC. The high levorotation of collagen is indicative of the unique polypeptide structure of that protein; and if part of the solution contained particles of a different structure, this dilution should have been reflected in the optical rotation. In addition, previous amino acid analyses (1) of intact EWCC showed an extremely high hydroxyproline content and a glycine content about one-third of the total residues. It is unlikely that these residues, which are so important to the collagen structure, could be limited primarily to one-half of the molecule.

It will be recalled that another significant difference between EWCC and TC is that EWCC contains a relatively large amount of carbohydrate. It is an interesting suggestion that this carbohydrate binds together the two halves of the EWCC molecule. If a single or double chain carbohydrate polymer linked the two EWCC halves, such a link would be broken first in the shearing process. To break through a collagen molecule which is composed of three parallel polypeptide chains, three covalent bonds would have to be ruptured. To break the carbohydrate link, only one or two bonds would have to be broken. Even if the link were triple, the covalent carbohydrate bonds are weaker than those in polypeptide chains; this in addition to the statistical probability that a rigid rod will break first in the center, even if it is equally strong at all points.

The observation that initiated the study of EWCC was that the gross fibrils in the cuticle are not striated as in other collagens. This may be explained in two ways, or a combination of them. The native type fibril owes its periodic striated appearance to the lateral alignment of TC molecules, all pointing in the same direction, but each staggered by one-quarter of its length with respect to most of its neighbors. This arrangement brings into lateral alignment the ends of the molecules every one-quarter of a molecule length, or about every 640 A (8). Such highly organized aggregation requires intimate contact between adjacent molecules. In EWCC, if carbohydrate were an integral part of the structure, it might preclude such close alignment. Or, if the two TC-like halves of the EWCC particle were attached to each other so that two similar ends were adjacent, this would mean that all the TC-like halves would not be aligned in the same direction, and that the repeating unit would be twice as long as in the case of vertebrate TC. If this were true, for the EWCC molecules to aggregate so that a 640 A period occurred, each lateral aggregation site would require the proper fit of more different groups than in TC; and if one of the sites of alignment were (say) bulkier than the others, this bulkier site would have to be incorporated into the repeating unit. At present, these explanations are highly speculative.

The possibility that reactive side groups are blocked by carbohydrate in intact EWCC and

that this carbohydrate is stripped off during shearing is under investigation. Preliminary results indicate that some of the carbohydrate is widely distributed throughout the macromolecule, but the sites of carbohydrate-protein bonds are not known with any certainty.

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