Collagenase from Rat Uterus. Isolation and Partial Characterization*

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ABSTRACT: An enzyme capable of degrading native collagen has been obtained from the medium of cultures of resorbing postpartum rat uterus, but not from tissue extracts. The enzyme is maximally active at neutral pH with little activity below pH 5 or above 8, requires Ca^{2+}, and is irreversibly inhibited by ethylenediaminetetraacetate but not by cysteine. At temperatures below 30° the enzyme catalyzes a small number of cleavages in the native collagen molecule with no loss in tertiary structure of the products. Three of these products have been identified by electron microscopy of adenosine triphosphate reconstituted crystallites (segment long spacing) as fragments of 75, 67, and 62% of the molecular length from the "A" end. Characterization of the smaller fragments is not yet accomplished. At 37° and neutral pH, uterine collagenase preparations degrade native collagen fibrils or molecules to peptides most of which are dialyzable.

Controlled degradation of collagen plays an important role in physiologic remodeling of animal tissues during growth and development as well as in repair and pathologic states. In recent years collagenolytic enzymes operative initially at neutral pH have been detected in cultures of a number of amphibian and mammalian tissues (Gross and Lapiere, 1962; Jeffrey and Gross, 1967; Grillo and Gross, 1967; Walker *et al.*, 1964; Eisen, 1967; Fullmer and Gibson, 1966; Riley and Peacock, 1967; Beutner *et al.*, 1966; Bennick and Hunt, 1967; Fullmer and Lazarus, 1967; Grillo *et al.*, 1968). Some of these collagenases have been isolated from the culture medium and partially characterized (Nagai *et al.*, 1966; Gross and Nagai, 1965; Sakai and Gross, 1967; Eisen *et al.*, 1968; Evanson *et al.*, 1967; Evanson and Krane, 1968). In two instances in which the products of reaction were characterized collagenolytic enzymes have been extracted directly from cells and tissue, one from human leucocytes (Lazarus *et al.*, 1968) and another from crab hepatopancreas (Eisen and Jeffrey, 1969).

In all cases the enzymes degrade native collagen fibrils, or molecules in solution, to relatively small polypeptide fragments at 37° and pH 7.6. At lower temperatures below the denaturation range of the substrate (*ca.* 20–28°), the attack is much more limited and specific. The collagen molecule is severed at a point one-fourth the length from the "B" end producing two conformationally intact fragments, TC^{A} which represents three-fourths the length from the "A" end, and TC^{B}, the remaining one-fourth. In the best described case, namely, the action of purified tadpole collagenase, the two fragments have been isolated and characterized by electron microscopy (Gross and Nagai, 1965), physical chemical analyses (Sakai and Gross, 1967), and amino acid composition (Kang *et al.*, 1966), and the terminal amino acids at the site of cleavage identified (Nagai *et al.*, 1964). Only three peptide bonds were severed per 300,000 molecular weight, presumably one in each chain about the same locus (Nagai *et al.*, 1964).

The enzyme obtained from cultures of postpartum rat uterus, briefly reported in 1967 (Jeffrey and Gross), was shown to differ in its mode of attack from the amphibian collagenase. This paper describes the isolation and characterization of the rat uterine collagenase.

Methods

Female Sprague–Dawley rats were killed less than 12-hr postpartum and both horns of the uterus excised under sterile conditions. They were washed several times with 10-ml portions of Dulbecco and Vogt's modification of Eagle's medium containing 300 units/ml of penicillin and streptomycin, after which the tissues were cut into uniform explants, approximately 2 ml^2, with sterile technique; 12–15 explants were placed in each of a series of disposable culture flasks (Falcon Plastic, Richmond, Calif.) containing 2.5 ml of medium. The flasks were gassed with 95% O_2–5% CO_2 and incubated at 37°. Medium was harvested daily, usually for 4 days, and fresh medium was added. The medium collected each day was augmented with 0.05 volume of 2 M Tris-HCl (pH 7.5), then dialyzed against distilled water, and lyophilized.

Collagenolytic activity was determined either viscometrically or by release of soluble radioactivity from ^{14}C-labeled reconstituted collagen fibrils (Nagai *et al.*, 1966). Viscosity was measured in Ostwald viscometers with flow time for water at 20° between 25 and 75 sec. Reconstituted native collagen fibrils were prepared from solutions of [^{14}C]glycine-labeled guinea pig skin collagen (specific activity 23,000 cpm/mg). A typical reaction mixture contained 150 μg of ^{14}C-labeled collagen fibrils, 15 μmoles of NaCl, 7.5 μmoles of Tris-HCl (pH 7.6), 450 mμmoles of $CaCl_2$, and from 5 to 500 μg of the crude enzyme protein in a final volume of 150 μl.

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The reaction mixture was incubated from 1 to 20 hr at 37° in 1-ml plastic centrifuge tubes tightly covered with Parafilm following which they were centrifuged at 20,000*g* for 10 min. A fixed aliquot usually 100 μl was counted in Bray's solution in a liquid scintillation counter. Controls were run using crystalline trypsin at a final concentration of 0.01%, and the reported data are counts per minute above that of the trypsin control.

Optical rotation measurements on reaction mixtures as a function of time of incubation were performed in a Rudolph photoelectric spectropolarimeter equipped with an oscillating polarizer. Temperature was maintained in the 1-dm jacketed cell by circulating water from a constant-temperature bath. Melting curves were obtained by following optical rotation at 367 mμ as the temperature was increased in steps of 1°/20-min increments.

Segment long-spacing aggregates of intact collagen and enzymatic products were prepared for electron microscopy according to Gross and Nagai (1965), stained with uranyl acetate, and examined in an RCA-EMU-3G electron microscope.

Electrophoresis of thermally denatured collagen in reaction mixtures on acrylamide gels was accomplished according to Nagai *et al.* (1964). The enzyme was inactivated by adding EDTA (10^{-2} M) before denaturation.

Calf skin and guinea pig skin collagen preparations and stock substrate solutions were prepared as described by Gross and Lapiere (1962).

Results

Enzyme Characteristics. Details of the purification of the enzyme will be deferred to a subsequent publication. Briefly, the activity has been purified approximately 15-fold by precipitation from concentrated culture medium with ammonium sulfate between 30 and 50% saturation. A further purification has been obtained by gel filtration on a column of Sephadex G-150. The enzyme emerges from the Sephadex column slightly behind the void volume. Although the enzyme solutions used in this study are impure, they are free from nonspecific proteolytic activities as indicated by their inability to degrade denatured casein at neutral pH and 37°. Unless otherwise specified, enzyme partially purified by ammonium sulfate precipitation was used in these experiments.

Partially purified enzyme solutions degrade collagen in suspensions of reconstituted native fibrils at pH 7.6 and 37°. A linear relationship between the amount of substrate degraded and time of incubation, and enzyme concentration is demonstrated in Figure 1a,b.

The dependence of enzyme activity on pH is shown in Figure 2. A fairly sharp peak of maximum activity is observed at pH 7.5 which peak nearly vanishes at pH 5.5.

The uterine collagenase is completely inhibited by 10^{-3} M EDTA and requires Ca^{2+} for activity as shown in Table I. Mg^{2+} and Zn^{2+} are only weakly active in replacing Ca^{2+}. After inhibition by EDTA, the addition of a large excess of calcium does not restore collagenolytic activity. This suggests that a second metal is required for enzymatic activity. The enzyme is inactivated by heating to 90° for 10 min and is not affected by 10^{-3} M cysteine, in contrast with the irreversible inactivation of tadpole collagenase by this amino acid.

Partially purified enzyme from postpartum uterine tissue

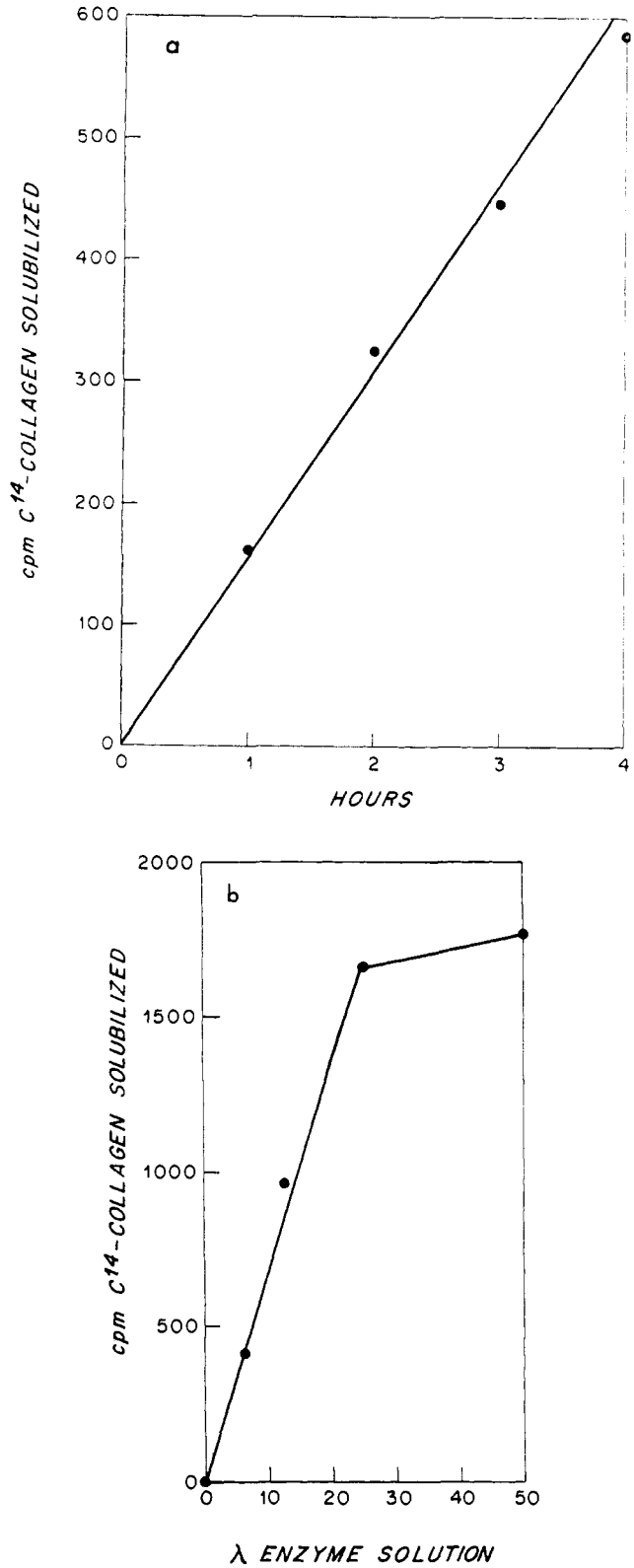

FIGURE 1: Release of radioactivity from [^{14}C]glycine-labeled collagen fibrils by uterine collagenase; (a) as a function of time and (b) as a function of enzyme concentration. Conditions of the assay system were as described in Methods. The enzyme solution, partially purified by $(NH_4)_2SO_4$ precipitation, contained 10 μg of protein μl. Incubation mixtures in (a) contained 100 μg of enzyme protein; incubation mixtures in (b) were incubated for 6 hr. Total radioactivity per incubation mixture = 2200 cpm.

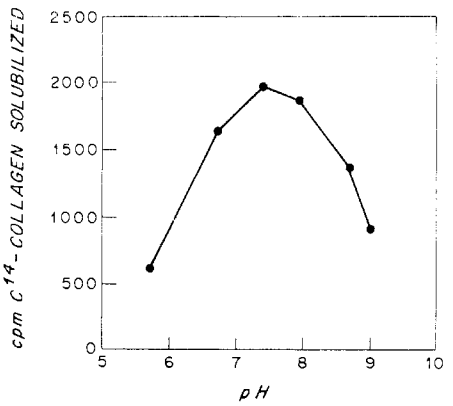

FIGURE 2: pH optimum of uterine collagenase, measured by the [^{14}C]collagen fibril assay. Conditions of the assay described under Methods. Buffers used to obtain the appropriate values were 0.2 M Tris–maleate (pH 5.7–7.9) and 0.2 M boric acid–KCl (pH 7.8–9.1). The final pH at each point was determined on mock reaction mixtures (no collagen or enzyme protein).

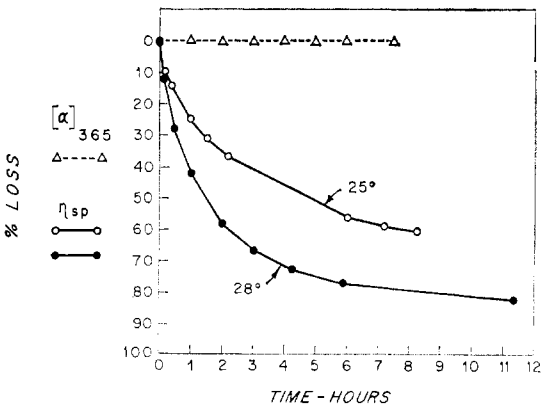

FIGURE 3: Viscosity and optical rotation measurements on uterine collagenase collagen reaction mixture. Initial collagen concentration, 0.05%, (η_{sp} = 2) in 0.05 M Tris–0.2 M NaCl–0.005 M $CaCl_2$ (pH 7.5). Enzyme protein concentration, 200 μg/ml of reaction mixture. Optical rotation was measured simultaneously with viscosity on an aliquot of the reaction mixture.

cultures reduced the viscosity of collagen solutions (pH 7.6) at 25 and 28°, temperatures which are well below the denaturation range (Figure 3). Activity could also be detected at 20°, but the rate of viscosity loss was comparatively slow. Both the rate of loss and fractional change were less at 25° than that at 28°.

Optical rotation measurements of the collagen substrate during the reaction (Figure 3) indicated no significant loss of helical structure during a drop of at least 50%, and in some experiments as much as 83%, of the specific viscosity.

The denaturation temperature midpoint (T_m) of the reaction products was approximately 7° lower than that of native collagen at pH 4 (Figure 4). In all cases where the reaction was less than complete the melting curve was diphasic, the latter section of which probably represents the remaining intact collagen molecule. T_m values of approximately 33–35° for the reaction products were obtained in several experiments in which the final viscosity ranged from 17 to 84% of control collagen which denatured with a T_m of 40°.

Disc electrophoretic patterns of thermally denatured reaction mixtures are shown in Figures 5 and 6. At viscosity losses up to 20% the appearance of a small number of new bands is seen. One appears below the normal α chains, another below the normal β component, and two fast-running bands appear near the buffer front. The pattern at this time in the reaction is identical with that obtained with tadpole collagenase and that from human skin (Eisen *et al.*, 1968) and rheumatoid synovium (Evanson *et al.*, 1967). As the

TABLE 1: Effect of Various Divalent Cations on the Activity of Dialyzed Uterine Collagenase.[a]

Metal Added	[^{14}C]Collagen Solubilized (cpm)
None	0
Ca^{2+}, 5×10^{-3} M	4750
Mg^{2+}, 5×10^{-3} M	1069
Zn^{2+}, 5×10^{-3} M	220
Mn^{2+}, 5×10^{-3} M	0
Cu^{2+}, 5×10^{-3} M	0

[a] Effect of various divalent cations on the activity of dialyzed uterine collagenase. Radioactive fibril assay. Enzyme solution dialyzed against 0.05 M Tris, 18 hr. Concentrations of metals added as the chlorides, represent final concentrations in the reaction mixtures. Total cpm added as [^{14}C]collagen = 7000.

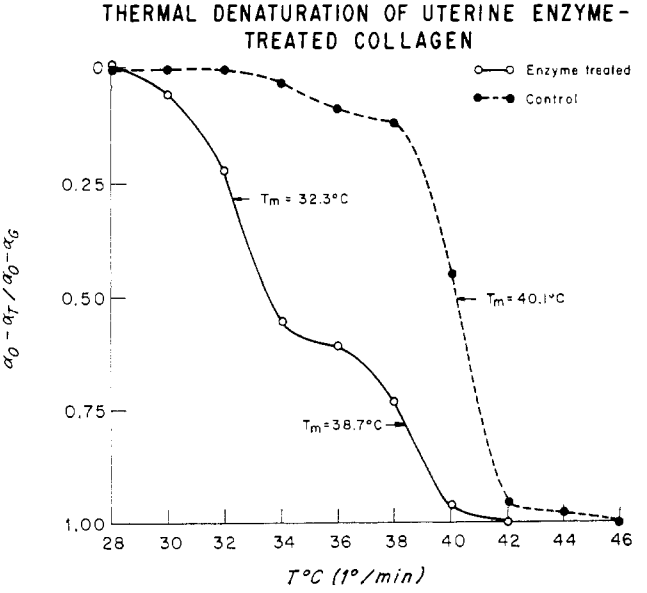

FIGURE 4: Thermal denaturation of normal collagen and collagen digested with uterine collagenase at 28° to a loss in η_{sp} of 60% (see Figure 3 for details). Denaturation was performed in 0.005 N acetic acid and followed in a Rudolph polarimeter at 365 mμ. The ordinate represents the fraction of the original optical rotation lost.

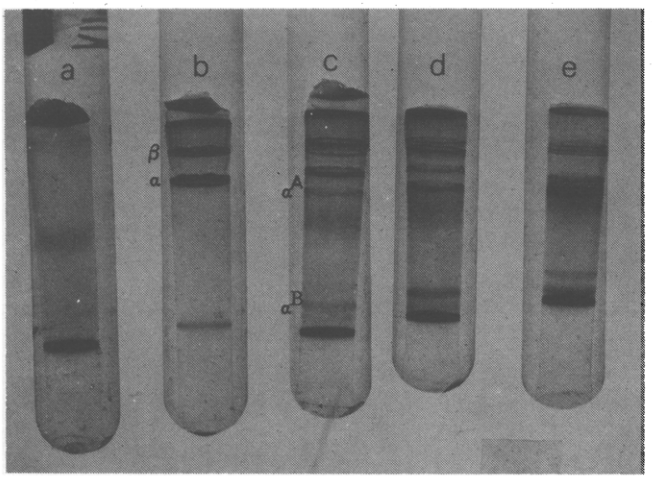

FIGURE 5: Acrylamide gel electrophoretic patterns of uterine collagenase degraded collagen reaction mixtures at 28°. (a) Enzyme control; (b) collagen control; (c) 10% viscosity loss; (d) 30% viscosity loss; (e) 60% viscosity loss; α refers to the single polypeptide chain and β to the cross-linked dimers of the α chains. These are obtained in the free random coil form after denaturation of collagen. α^A and β^A refer to modified α and β components whose A (NH_2 terminal) ends are intact. α^B, similarly, denotes modified α chains whose B (COOH terminal) ends are intact. Lowest band in each gel is solvent front.

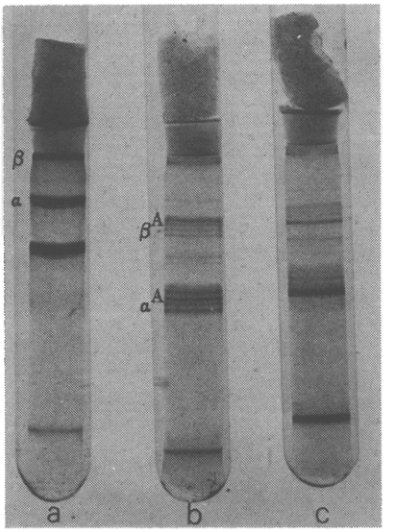

FIGURE 6: Acrylamide gel electrophoretic patterns of uterine collagenase degraded collagen; (a) collagen control; (b) 60% viscosity loss; (c) 70% viscosity loss. See Figure 5 for nomenclature of modified components.

reaction proceeds, however, a more complicated pattern of products appears (Figure 6). At least two new components are seen below each of the original new bands. This more complicated display of new fragments is maintained qualitatively unchanged as the reaction proceeds. A progressive loss of the original α chains and β components is observed, paralleled by an increase in concentration of the modified components. A gradual increase in the amount of the fastest moving component in the modified α and β region is seen (Figure 6). The reaction products at 25° are identical with those at 28° at equivalent viscosity losses. The two fast-moving bands in the α^B region (Figure 5) are present throughout the reaction at either temperature and no dialyzable material is produced. Electrophoretic patterns of reaction mixtures incubated at temperatures between 33 and 37° show a single dense band at the solvent front and 67% of the reaction products are dialyzable at 37°. Reaction mixtures at 28°, inhibited by EDTA and then elevated to temperatures between 33 and 37°, did not produce these fast-moving small peptides. Thus, the production of these small fragments is the result of further enzymatic attack rather than thermal lability.

Electron microscopic examination of ATP-precipitated segment long spacing from reaction mixtures revealed the presence of segments of different lengths, depending upon the extent of the reaction. Measurements of the loss in molecular length were made by comparing the length of the whole segment (AB′) with the position of a well-defined landmark within that segment, in this case, the δ_3 band of Hodge and Schmitt (1960). The mean AB:Aδ_3 ratio for native collagen (TC) is 2.35. The mean ratio of the three-quarter-length segment, TC^A_{75}, produced by tadpole collagenase is 1.75 (Gross and Nagai, 1965). Segments whose AB′:Aδ_3 ratio is 1.75 are readily obtained from uterine collagenase reaction mixtures, particularly at early time periods. In addition to TC^A_{75} segments, two shorter molecular species which begin to appear as the viscosity falls below 60% of the controls have been identified. The segment long-spacing crystallites consisting of fragments from the "A" end, have ratios of 1.53 and 1.45, representing molecular lengths of about 67 and 62% of native collagen. These will be referred to as TC^A_{67} and TC^A_{62}, respectively. Figure 7 illustrates the distribution of lengths of segment long spacing seen in various reaction mixtures and the shaded area allows comparison with the TC^A fragments measured from reaction mixtures of collagen and tadpole collagenase (Gross and Nagai, 1965). Small discrete differences in molecular length, so evident from inspection of the band pattern, are not separately resolved in the distribution curve. The

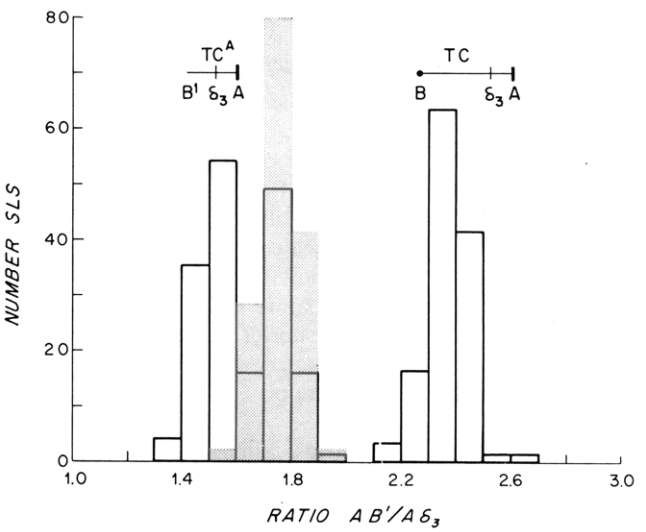

FIGURE 7: Distribution pattern of the molecular lengths of collagen fragments produced by uterine collagenase. Shaded bars represent distribution curve for fragments (TC^A_{75}) produced by the action of tadpole collagenase. Bars on the right represent the distribution obtained for normal collagen (TC).

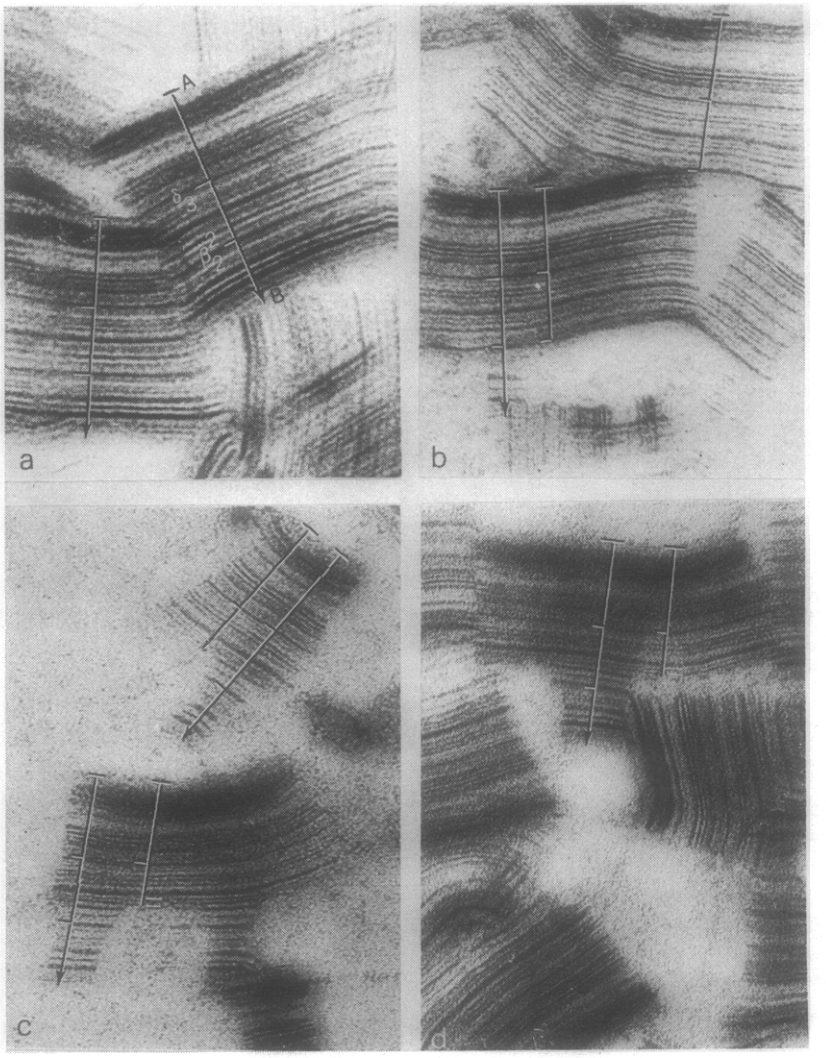

FIGURE 8: Electron micrographs of segment long spacings of collagen fragments produced by the action of uterine collagenase: (a) normal collagen segments (TC), with the δ_3 and $B_2{}^2$ (TC^A_{75}) bands marked for reference; (b) TC^A_{75} segment hybridized to a small amount of normal TC, represented by the full-length arrow; (c) in the upper right corner a hybrid of normal collagen and TC^A_{67}. The distal bands of the shortened segments are marked with white lines. In the lower left is displayed a hybrid of normal collagen and TC^A_{62}; the distal bands are again marked in white. (d) A multiple hybrid containing normal collagen (full arrow), TC^A_{62} (white lines) and small areas of TC^A_{67}, visible in places below bands of TC^A_{62}. Magnified 70,000 times.

distinguishing characteristics are clearly seen in the electron micrographs shown in Figure 8. Figure 8a illustrates segment long spacing of normal intact collagen (TC), and 8b shows TC^A_{75}, which in one region indicated by the complete arrow is coprecipitated with a small amount of TC. The distal band in TC^A_{75} is the B^2_2 in the nomenclature of Hodge and Schmitt (1960). Figure 8c,d shows the shorter 67 and 62% fragments, TC^A_{67} and TC^A_{62} also coprecipitated with intact molecules. In some preparations where digestion is complete there are no hybrids.

One-quarter-length segments, similar to TC^B_{25} obtained from tadpole enzyme reaction mixtures have been seen on rare occasions in the form of stacks of segments and occasionally as individuals. Figure 9 illustrates an extended ribbon of such a "stack" stained with uranyl acetate. It is difficult to match up the band pattern in the ribbon precisely with the known structure of TC^B_{25}, because of a small amount of regular overlap of the individual TC^B_{25} fragments within the polymer. Disc electrophoresis indicates that the small pieces are always present and there is no indication of modification or additional fragments. Modifications at the "A" (NH_2 terminal) end of the molecule have not been observed in these studies. Tantalizingly, other very short, well-structured segments have occasionally been seen, but as yet the site of origin of these small fragments in the collagen molecule have not been localized.

Discussion

The characteristics of the catalytic activity of the uterine collagenase are similar in many ways to that of the tadpole enzyme. Both have pH optima in the neutral range and are nearly inactive below pH 6.0. Both are inhibited by EDTA; uterine enzyme, however, is irreversibly inhibited by the chelating agent whereas tadpole collagenase is not. Cysteine inhibits tadpole enzyme activity but not that of the uterus. Both

enzymes have high molecular weights (>50,000) although the uterine enzyme may be somewhat larger. Both enzymes catalyze an attack at a single locus on the collagen molecule one-quarter of the full molecular length from the "B" end at temperatures below that of the substrate denaturation. The uterine enzyme, however, is also capable of catalyzing further breaks in undenatured TC^{A}_{75} whereas tadpole collagenase is not. The possibility of multiple enzyme activities in the semipurified uterine preparation is not yet fully ruled out. There is no loss of helical content, but there is a significant decrease in the stability to heat of the fragments in solution. Both tadpole and uterine enzymes are capable of further degrading the substrate to much smaller polypeptides at 37° after thermal denaturation of the products of initial cleavage.

It is not possible at this time to unequivocally impute the complete degradation of the collagen molecule at 37° to a single enzyme in our semipure preparations. It is possible that very low levels of other proteolytic enzymes are present in these enzyme solutions. The fact that EDTA prevents further degradation at 37°, of products formed at 25 or 28°, and that no measureable caseinolytic activity is present in the preparation suggests, however, that the collagenolytic enzyme itself does act upon the denatured products of initial cleavage.

It appears, then, that there is a small number of sites on the native collagen molecule with different degrees of susceptibility to attack by the uterine enzyme. At physiologic temperature the large products of the initial cleavages denature, exposing large numbers of susceptible bonds, since at 37° collagen molecules in solution or in fibrils are rapidly degraded to small dialyzable peptide fragments. It is probable that the reactions observed at lower temperatures are identical with the initial reactions catalyzed at physiologic temperature, except that the rates are slowed sufficiently at lower temperature for the processes to be separated. There may be three highly localized regions of differing susceptibility to enzymatic attack in the native molecule, which might depend upon sharply localized loosening of the helix; the extensive digestion of completely denatured collagen is suggestive of this.

Recently Kühn and Eggl (1966) have reported on the action of *Cl. histolyticum* collagenase on collagen at low temperature, which contrasts sharply with that of vertebrate collagenases. The earliest activity of the bacterial enzyme is the removal of a small section from the "A" end of the collagen molecule, and the subsequent attack on the "B" end differs significantly from that catalyzed by uterine, tadpole, and other mammalian enzymes thus far isolated.

The uterine collagenase, in common with those from human synovium and skin and the amphibian collagenases, could not be extracted directly from the tissue, but was found only in the medium of living cultures; freeze–thawing the tissue abolished activity. Thus, it would appear that these enzymes are synthesized *de novo*, perhaps as needed, and not stored for later release.

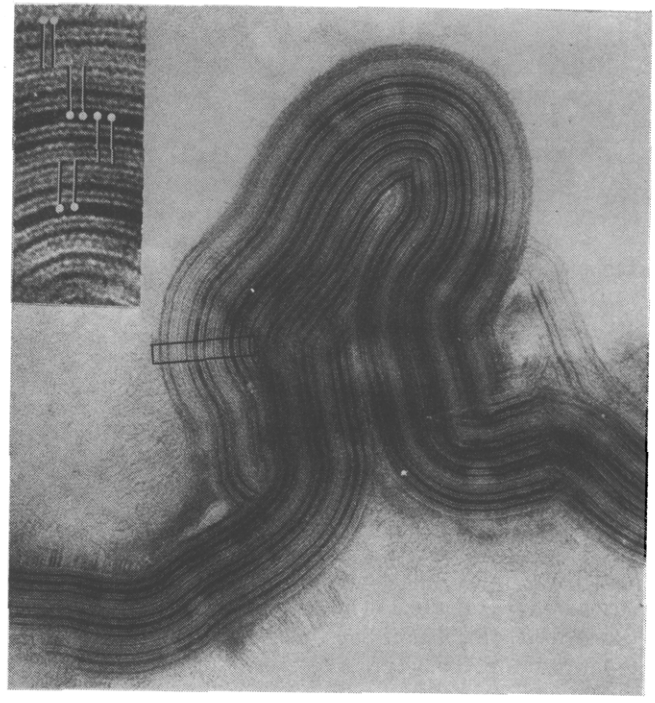

FIGURE 9: Electron micrograph of oriented ribbons of segment long spacings formed from fragments of the collagen molecule at the "B" end, designated TC^{B}. Stained with uranyl acetate. Magnification 35,000 X. Insert enlarged from region outlined, 100,000 X.

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