Studies on collagenase from rheumatoid synovium in tissue culture

John M. Evanson, … , John J. Jeffrey, Stephen M. Krane

J Clin Invest. 1968;47(12):2639-2651. https://doi.org/10.1172/JCI105947.

Research Article

Fragments of synovium from patients with rheumatoid arthritis survive in defined tissue culture medium in the absence of added serum and, after 3-4 days, release into the medium enzyme capable of degrading undenatured collagen. Maximal activity is observed at pH 7-9 but the enzyme is inactive at pH 5. At temperatures of 20° and 27°C, collagen molecules in solution are cleaved into 3/4 and 1/4 length fragments with minimal loss of negative optical rotation, but with loss in specific viscosity of approximately 60%. Above 30°C the fragments begin to denature and denaturation is complete at 37°C. If the enzyme is not inhibited at this stage the large fragments are broken down further to polypeptides of low molecular weight. Reconstituted collagen fibrils and native fibers at 37°C are cleaved to the low molecular weight fragments, although the fibrils are resistant to breakdown at lower temperatures (20°-27°C). It is proposed that the production of such an enzyme by inflamed and proliferating rheumatoid synovium may be responsible for some of the destruction of collagenous structures that accompanies rheumatoid arthritis.

Studies on Collagenase from Rheumatoid Synovium in Tissue Culture

JOHN M. EVANSON, JOHN J. JEFFREY, and STEPHEN M. KRANE

From the Department of Medicine, Harvard Medical School, and Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114

ABSTRACT Fragments of synovium from patients with rheumatoid arthritis survive in defined tissue culture medium in the absence of added serum and, after 3–4 days, release into the medium enzyme capable of degrading undenatured collagen. Maximal activity is observed at pH 7–9 but the enzyme is inactive at pH 5. At temperatures of 20° and 27°C, collagen molecules in solution are cleaved into 3/4 and 1/4 length fragments with minimal loss of negative optical rotation, but with loss in specific viscosity of approximately 60%. Above 30°C the fragments begin to denature and denaturation is complete at 37°C. If the enzyme is not inhibited at this stage the large fragments are broken down further to polypeptides of low molecular weight. Reconstituted collagen fibrils and native fibers at 37°C are cleaved to the low molecular weight fragments, although the fibrils are resistant to breakdown at lower temperatures (20°–27°C). It is proposed that the production of such an enzyme by inflamed and proliferating rheumatoid synovium may be responsible for some of the destruction of collagenous structures that accompanies rheumatoid arthritis.

This is publication No. 460 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities.

Dr. Evanson is a Travelling Fellow of the Medical Research Council of Great Britain. His present address is Division of Metabolism, Department of Medicine, The Royal Infirmary, Manchester 13, England. Dr. Jeffrey's present address is Division of Dermatology, Department of Medicine, Washington University School of Medicine, St. Louis, Mo.

Received for publication 8 July 1968.

INTRODUCTION

Native collagen is relatively insusceptible to the action of the common proteolytic enzymes and evidence is beginning to accumulate that collagen breakdown in animal tissues is the result of the action of specific collagenases elaborated by connective tissue cells. Collagenolytic activity has been detected in a number of situations (1, 2), but apart from the amphibian collagenase (3, 4), the rat uterine collagenase (5), and the collagenase from human skin (28), few of these enzymes have been shown to attack native collagen at physiologic pH and temperature, and information on their mode of action is sparse. We have previously reported the detection and partial characterization of a specific collagenase in the tissue culture medium of rheumatoid synovial tissue (6), and we report here further observations on this human collagenase.

METHODS

Preparation of collagen. Radioactively labeled and unlabeled collagen was purified from the skin of actively growing guinea pigs of approximately 200 g body weight. Collagen labeled with ^{14}C-glycine was obtained by injecting the guinea pigs with 1.0 μc of 2-^{14}C-glycine/g body weight intraperitoneally 6 hr before killing. Neutral salt-soluble collagen, extractable in 0.5 M NaCl was purified by the method of Gross (7). Acid-soluble collagen was obtained by extracting the residue, after two salt extractions, with 0.1 M acetic acid. The collagen preparations were lyophilized and stored in a dessicator at −20°C until use. Solutions of collagen were prepared by dissolving the collagen in cold potassium phosphate buffer, ionic strength 0.4, pH 7.6, followed by dialysis against 0.4 M NaCl overnight at 4°C. A typical prepara-

tion of purified labeled neutral salt-soluble collagen had a specific activity of approximately 75,000 cpm/mg collagen. This was diluted with varying amounts of nonlabeled collagen in the assays for collagenolytic activity. Purified embryonic calf skin collagen (a gift of Dr. M. L. Tanzer) was used in some experiments involving viscometry.

Tissue culture assay for collagenolytic activity. Collagenolytic activity of synovium was examined by incubating fragments of tissue on reconstituted ^{14}C-labeled collagen gels in Leighton tubes (fluid chamber measuring 1 × 2 cm) for 3–5 days (4). Aliquot portions of 0.5 ml of approximately 0.1% labeled collagen solution, made up in mammalian Tyrode's solution containing added amino acids, penicillin, and streptomycin (3, 28), were dispensed under sterile conditions into the tubes and incubated at 37°C overnight to produce a gel. Fragments of tissue obtained at operation (usually synovectomy) were planted onto the gels, the tubes gassed with 95% O_2 and 5% CO_2, and incubated for 3–5 days at 37°C. Each Leighton tube contained 6–8 fragments of synovium, each approximately 2–3 mm in greatest dimension. At the end of this period the contents of the tubes were centrifuged at 20,000 *g* and an aliquot portion of the supernatant solution was added to a modified Bray's solution (10). Total soluble radioactivity released from the collagen gels was measured in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ohio) and was used as a measure of collagenolysis. In a typical experiment 6–8 Leighton tubes were used to assay synovial tissue, one tube was incubated without tissue, and 25 μg of purified trypsin was added to a final tube as a control for nonspecific proteolytic activity.

Preparation of crude enzyme solution. Rheumatoid synovial tissue, obtained at operation from patients with definite or classical rheumatoid arthritis (8), was immersed in tissue culture fluid in sterile Petri dishes for periods less than 1–2 hr before planting. Fragments of the synovial lining layer (approximately 5.0 mm in greatest diameter) were dissected free from the capsule and distributed under sterile conditions into 2.5 ml of Dulbecco's modified Eagle's medium containing glutamine, streptomycin, and penicillin without serum, in 30-ml disposable tissue culture flasks. The flasks were gassed with a mixture of 95% O_2 and 5% CO_2, sealed, and incubated at 37°C. Each flask contained 6–10 fragments of synovial tissue. The medium was harvested and replenished daily for 5–8 days. In some experiments all the harvested medium was pooled before further manipulation; in other experiments each day's medium was dealt with separately. The medium was dialyzed exhaustively against distilled water for 24–36 hr and then lyophilized. The crude enzyme powder was taken up in 0.1 M Tris-HCl, pH 7.6, containing 0.001 M $CaCl_2$ and centrifuged at 15,000 *g* to remove undissolved protein.

Viscometry. Viscosity measurements were made using Ostwald viscometers with water flow times at 20°C of 20–25 sec or 60–70 sec. The reaction mixture was a solution containing 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.6, and 0.0002 M $CaCl_2$ and collagen in a concentration of approximately 0.1%.

^{14}C-labeled collagen fibril assay (microgel assay). Collagenolytic activity was also assayed by measuring the release of soluble radioactive products from ^{14}C-glycine-labeled reconstituted fibrils, by a modification of the method of Nagai, Lapiere, and Gross (9). In most instances, reconstituted collagen fibrils were prepared by incubation overnight at 37°C of 50–100 μl of the labeled collagen solution (corresponding to 100–200 μg of collagen). Just before incubation, buffer (0.1 M Tris-HCl, pH 7.5, 0.002 M $CaCl_2$) with or without enzyme was added to a total volume of 250 μl, and the collagen fibrils dispersed. At the end of the incubation period, the tubes were centrifuged at 20,000 *g* and the radioactivity measured in an aliquot portion of the supernatant solution as described previously. In experiments designed to investigate the effect of temperature on the enzymatic digestion of fibrils, the gels were prepared by incubation of the collagen solution at 37°C for 72 hr. This longer period of incubation was used to minimize the spontaneous solubility of the fibrils when subsequently incubated with enzyme at lower temperatures.

Measurements of optical rotation. Measurements of optical rotation during enzymatic digestion of collagen at 27°C and the subsequent determination of the denaturation temperature of the products were performed at 240 mμ using a spectropolarimeter (Model 60, Carey Electronic Engineering Co., Springfield, Ohio), with a cell of 0.1 cm path length, jacketed to permit control of temperature.

Gel filtration. Molecular sieving of labeled reaction products was carried out with columns (1 × 35 cm) of Sephadex G-75 equilibrated with 5 M LiCl containing 0.01 M Tris-HCl, pH 7.4, at room temperature. Fractions of approximately 1.0 ml were collected every 2 min. Human gamma globulin, cytochrome *c*, and ^{3}H-proline were used as column markers. Optical density of the effluent at 280 mμ was monitored continuously with a recording spectrophotometer (Gilford Instrument Company, Oberlin, Ohio), and aliquot portions of the collected fractions were added to a modified Bray's solution (10) and assayed for ^{14}C and ^{3}H.

Chemical methods. Hydroxyproline was determined either by the method of Prockop and Udenfriend (11) or that of Bergman and Loxley (12). Protein was estimated by the method of Lowry, Rosebrough, Farr, and Randall (13), using crystalline bovine serum albumin as a standard.

Enzymes. Crude clostridial collagenase (Worthington Biochemical Corp. Freehold, N. J.) was purified by the method of Seifter, Gallop, Klein, and Meilman (14). Twice recrystallized trypsin was obtained from Worthington Biochemical Corp.

Disc gel electrophoresis. Disc electrophoresis in polyacrylamide gels of reaction products of collagen and synovial enzyme was carried out by the method described by Nagai, Gross, and Piez (15).

Electron microscopy. Solutions of collagen fragments resulting from enzymatic action were dialyzed against

0.05 M acetic acid and precipitated as segment long spacings (SLS) by the addition of adenosine triphosphate to a final concentration of 0.3%. They were positively stained with 1.0% phosphotungstic acid (pH 3.5) and examined in a RCA EMU 3G electron microscope (16). The formation of SLS requires the triple helical structure of the undenatured (or renatured) molecule (16). The length of the SLS is a measure of the length of the collagen molecule.

Histology. Representative samples of synovial tissue were taken before, during, and at the end of the period of culture and sections stained with hematoxylin and eosin for light microscopic examination.

Caseinolytic activity. Nonspecific proteolytic activity of collagenase and trypsin solutions was measured by the ability to digest casein, using the method of Kunitz (17).

RESULTS

Collagenolytic action of rheumatoid synovial tissue in culture. Synovial tissue from 11 of 13 subjects with rheumatoid arthritis, when cultivated in Leighton tubes, produced lysis of collagen gels in excess of trypsin-treated controls. Tissue was obtained at time of synovectomy, most frequently from the knees, wrists, and metacarpophalangeal joints. Results of a typical experiment with synovial fragments from one patient with rheumatoid arthritis are illustrated in Fig. 1. All samples of tissue examined before planting showed the characteristic changes of rheumatoid synovitis with proliferation of synovial lining cells, prominent vessels, and a variable infiltration of the subsynovial lining layer with lymphocytes and plasma cells. Polymorphonuclear leukocytes were rarely

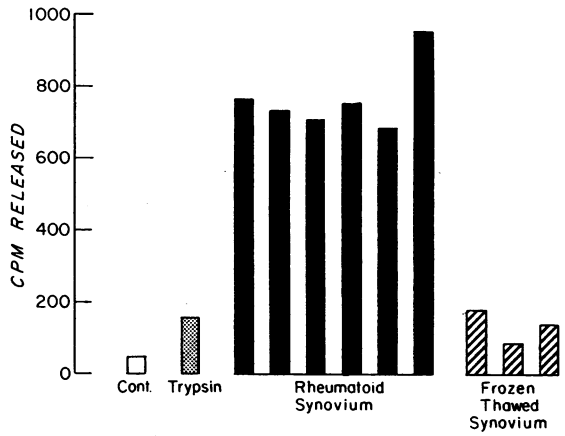

FIGURE 1 Lysis of ^{14}C-glycine-labeled collagen gels in Leighton tubes. Each bar represent one Leighton tube containing labeled collagen gels (1900 cpm). The solid bars represent Leighton tubes containing 6–8 fragments of synovium from the knee joint of a single patient with classical rheumatoid arthritis. Incubation period was 72 hr at 37°C. The tube labeled trypsin contained 25 μg of crystalline trypsin. The ordinate refers to the radioactivity released into the supernatant solution after centrifugation.

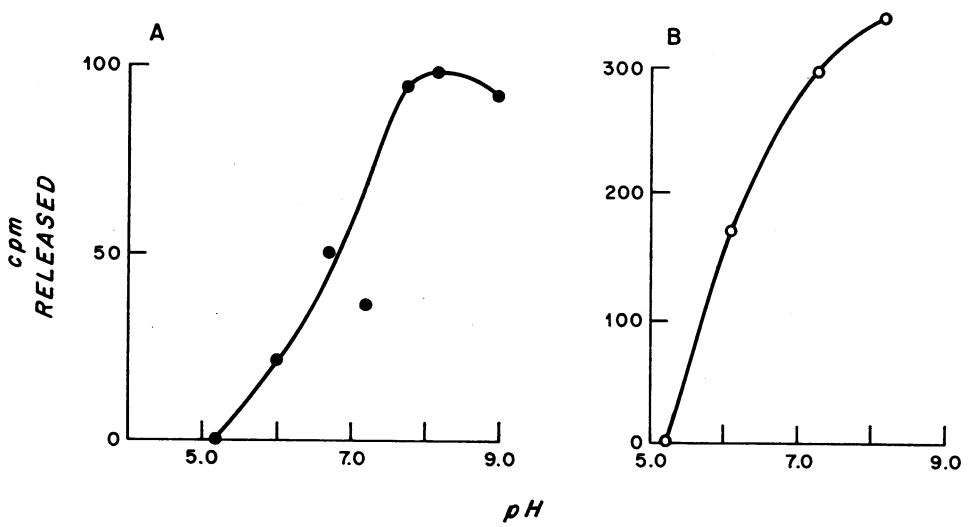

FIGURE 2 Collagenolytic activity as a function of pH. Microgel assay was performed as described in the text. In *A*, each gel contained 1000 cpm and the same volume of crude enzyme solution. Values shown are corrected for radioactivity released from gels at each pH in the absence of enzyme. Buffers were 0.1 M Tris-maleate (pH 5.2–7.2) and 0.1 M Tris-HCl (pH 7.8–9.0). In *B*, enzyme prepared from the synovium of a different patient was incubated with gels containing 680 cpm and similar buffers for the same period (2.5 hr).

seen. After culture, few inflammatory cells remained but hypertrophy of the synovial lining cells, endothelial cells, and other mesenchymal cells was seen. In those instances in which negligible lysis of the gels occurred, histological examination after the period of incubation showed little viable-appearing tissue. Tissue which was rapidly frozen and thawed before planting produced no collagenolysis, and on the few occasions when bacterial contamination occurred no collagen was solubilized.

Collagenolytic action of tissue culture medium. Collagenolytic activity was detected by several methods in the dialyzed, concentrated tissue culture media. Using the ^{14}C-labeled collagen fibril assay at pH 7.6, we showed radioactivity released to be directly proportional to the amount of synovial enzyme added and to the duration of incubation at 37°C (6), thus establishing the validity of the assay for comparing activities of different enzyme solutions. The pH dependence of the enzyme was determined using the labeled fibril assay. Fig. 2 shows the results of two experiments using different enzyme preparations. There is a broad pH optimum above pH 7.0 with no activity demonstrable at pH 5.3. Enzymatic activity was abolished by heating to temperatures above 70°C for 5 min.

Crude enzyme solutions prepared, as described, from the noncollagen-containing tissue culture

TABLE I

Comparison of Actions of Trypsin and Synovial Collagenase on Collagen and Casein

Enzyme	Protein added	Collagenolysis	Protein added	Caseinolysis
	μg	cpm*	μg	OD 280mμ
Synovial collagenase	54	599	135	0.051
	135	1385	270	0.063
Trypsin	2	20	1	0.272
	10	34	2	0.510

Collagenolysis assayed using microgels of ^{14}C-glycine–labeled collagen fibrils, each tube containing approximately 2500 cpm and incubated with enzyme at pH 7.4 for 3 hr at 37°C. Caseinolysis assayed using the procedure of Kunitz (17) with incubations at 37°C for 1 hr.

* cpm released over control.

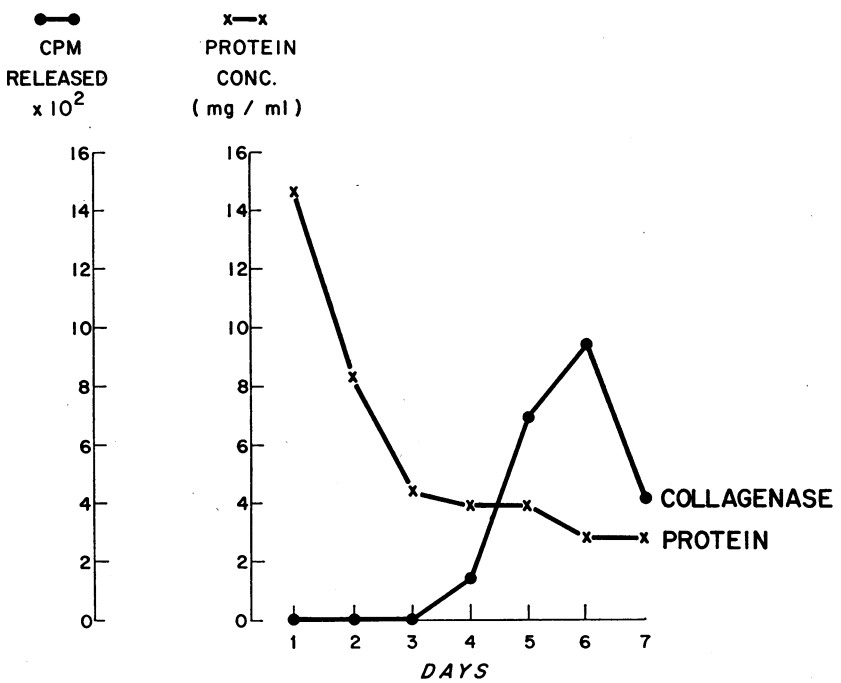

FIGURE 3 Collagenolytic activity of culture medium as a function of time of incubation of synovial fragments. The media from 29 flasks, each containing 6–8 fragments of synovium from a patient with rheumatoid arthritis, were harvested daily. Each day's harvest was pooled, dialyzed, lyophilized, and dissolved in 3.0 ml buffer. Aliquots of 0.1 ml were assayed in the microgel system (980 cpm/gel).

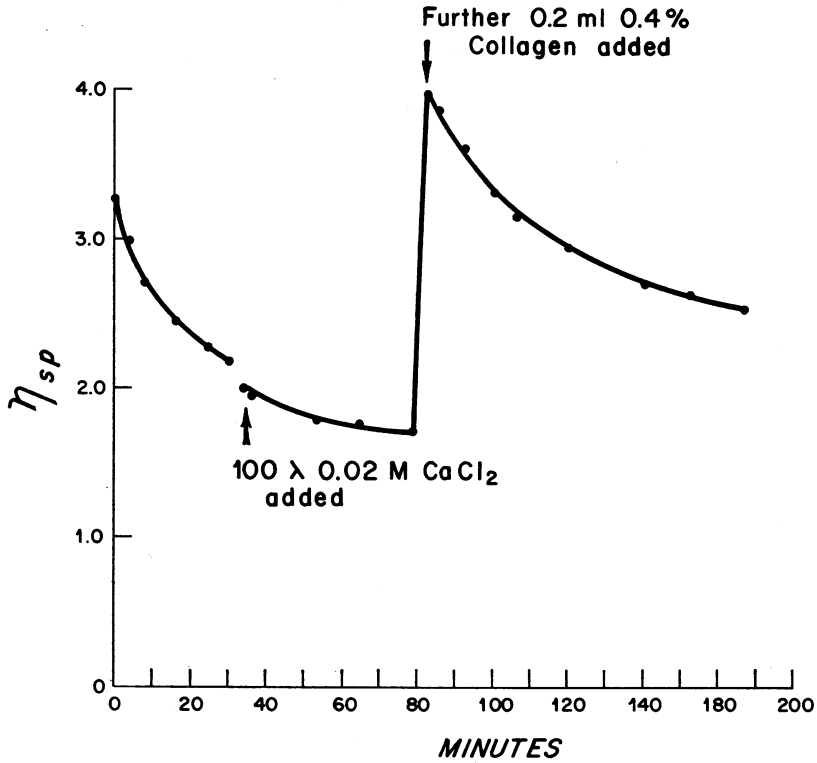

FIGURE 4 Collagenolytic activity as determined by viscometry. The viscometer contained initially 1.0 ml total volume of solution consisting of 0.2 ml of approximately 0.4% calfskin collagen, 0.3 ml buffer (pH 7.6), and 0.5 ml crude enzyme solution in the same buffer, incubated at 27°C. After 34 min, 0.1 ml of 0.02 M $CaCl_2$ was added. After 82 min, another 0.2 ml of 0.4% collagen solution was added. Ordinate indicates specific viscosity (η_{sp}).

medium, showed marked collagenolytic activity but negligible nonspecific proteolytic action as judged by the ability to digest casein. Table I compares the action of two batches of collagenase with that of trypsin at two concentrations. At these levels trypsin showed trivial collagenolytic activity as measured by the ^{14}C-fibril assay but significant caseinolytic activity. The converse was true of the collagenase solution. Different preparations of crude collagenase showed various degrees of nonspecific proteolytic activity but always only a minor fraction of that shown by a concentration of trypsin virtually inactive against reconstituted collagen fibrils.

In those experiments where each day's tissue culture medium was processed separately the time course of production of collagenolytic activity in the in vitro system was studied. Enzyme activity was usually first detectable on the 3rd and 4th day of culture, reached its maximum on the 5th or 6th day, and was still present at 8 days. Fig. 3 shows the result of one experiment in which collagenase activity and total protein concentration were measured. Maximum collagenolytic activity appeared at a time when total protein content had fallen from initial high levels. In contrast, in other experiments not shown, the amount of nondialyzable hydroxyproline peptides present in the medium paralleled approximately the changes in collagenase activity.

The specific viscosity of collagen solution of approximately 0.1% concentration was reduced by 55–70% when incubated with the synovial enzyme at 27°C. Addition of further collagen at the end point of the reaction resulted in the expected immediate increase in viscosity and was followed by a further fall in viscosity (Fig. 4), whereas addition of more synovial enzyme produced no effect. These results suggested that the limited reduction in viscosity produced by the synovial en-

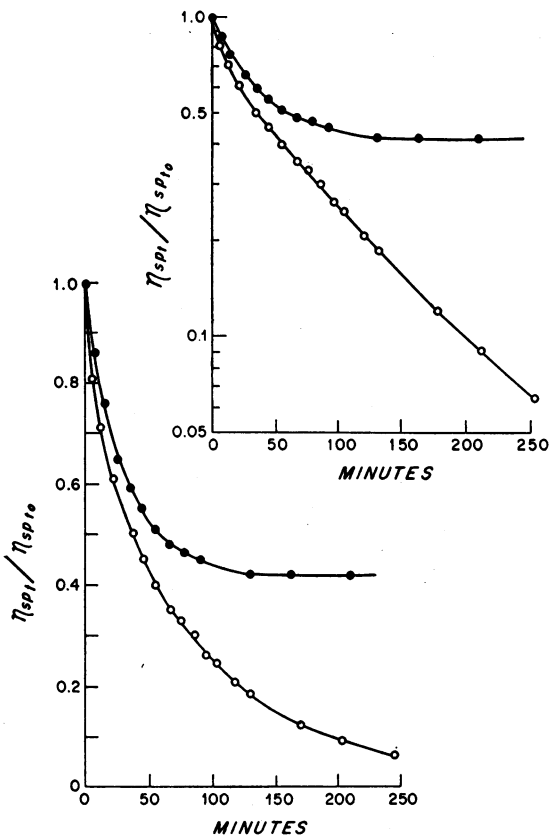

FIGURE 5 Effects of clostridial collagenase and synovial collagenase on solutions of collagen at 27°C. Conditions are the same as those shown in Fig. 4. In the viscometer depicted by the curve connecting closed circles, crude synovial enzyme (1.5 mg protein) was added at time zero. In the second viscometer (open circles) 10 μg purified clostridial collagenase was added. On the ordinate is plotted the fractional change in specific viscosity ($\eta_{sp_t}/\eta_{sp_{to}}$) at each measured point in time. In the inset the same data are plotted on a semilogarithmic scale.

zyme was not due simply to inactivation of the enzyme during the course of the incubation. In Fig. 5 the fall in viscosity resulting from the action of purified clostridial collagenase on a collagen solution (approximately 0.1%) at 27°C is compared with that due to synovial enzyme. The final loss of viscosity resulting from the bacterial enzyme was always greater than that produced by synovial enzyme, even when the initial rates of decrease in viscosity were similar. Furthermore, at the end point of the reaction between synovial enzyme and collagen, bacterial enzyme reduced the viscosity still further until it approached that of the solvent alone (6).

Ethylenediaminetetracetic acid (EDTA), when added in a concentration sufficient to chelate all calcium present (greater than 0.002 M), completely inhibited the synovial collagenase both in the fibril assay and as measured viscometrically. The addition of excess calcium to the EDTA-inhibited system did not restore enzyme activity.

Polarimetry indicated that the loss of viscosity due to the action of the synovial enzyme at 27°C was accompanied by no significant loss of optical rotation. For example, in Fig. 6 are shown the results of one experiment in which measurements of viscosity and optical rotation at 240 mμ were obtained on identical reaction mixtures of calfskin, collagen and enzyme. Decrease in specific viscosity of approximately 60% was associated with a de-

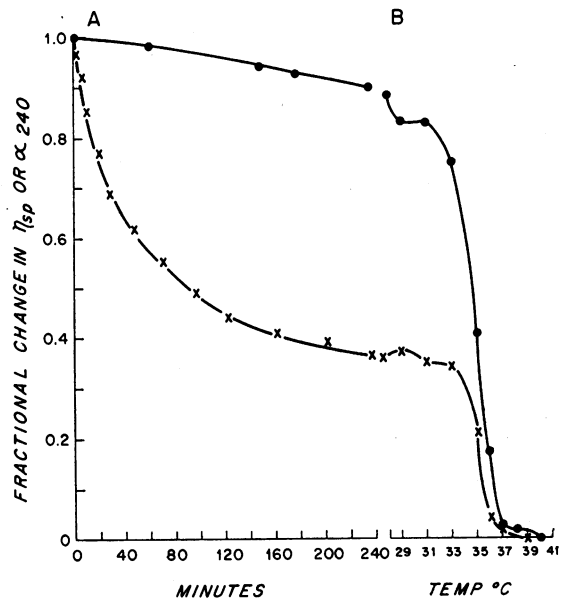

FIGURE 6 Effect of synovial collagenase on collagen solutions as assayed by simultaneous measurement of change in viscosity and optical rotation. Conditions for this experiment are similar to those shown in Fig. 4. Synovial enzyme, buffer, and collagen solution were mixed at 2°C, following which 1.0 ml was added to the viscometer and an additional aliquot portion added to the cell of a Carey 60 spectropolarimeter and measurements of optical rotation made at 240 mμ with the cell temperature brought to 27°C at point *A*. The viscometer was also maintained at 27°C. Just before point *B*, EDTA was added simultaneously to both the polarimeter cell and viscometer and the temperature raised. On the ordinate is plotted the fractional change in specific viscosity (η_{sp}) (×—×) and negative optical rotation at 240 mμ (α_{240}) (●—●).

crease in negative optical rotation of only 7%. When the reaction was complete, EDTA was added to a final concentration of 0.01 M to inhibit the enzyme and the temperature was elevated stepwise in increments of 1° or 2°C, maintaining each level for 10–15 min. Raising the temperature in this manner resulted in simultaneous loss of residual viscosity and optical rotation with a midpoint melting temperature (T_m) of approximately 35°C. The effect of trypsin on the products of collagen digestion by synovial collagenase at temperatures below denaturation temperature could be studied viscometrically (Fig. 7). When the reaction of calfskin collagen and synovial collagenase at 27°C was complete, as indicated by no further fall in viscosity, EDTA was added to a final concentration of 0.01 M, followed by the addition of 100 μg trypsin. The temperature was then elevated stepwise in increments of 1°C, maintaining each level for 10 min, and measurements of viscosity continued. The presence of the trypsin resulted in a shift of the melting curve to the left (Fig. 7) with a lowering of the T_m of approximately 3.5°C. However, at 27°C, the products of digestion by synovial enzyme were still resistant to cleavage by trypsin as shown by the minimal loss in viscosity.

The observations that at 20–27°C (i.e. below the denaturation temperature for collagen in solution) the synovial collagenase reduced the viscosity of collagen solutions only 60% with minimal decrease in negative optical rotation (i.e. the helicity was maintained) suggested that this enzyme was producing a limited cleavage of the collagen molecule similar to that produced by amphibian enzyme (9, 16). To explore this further, segment long spacings (SLS) were prepared from 20°C reaction mixtures of collagen and enzyme and examined with the electron microscope. These showed

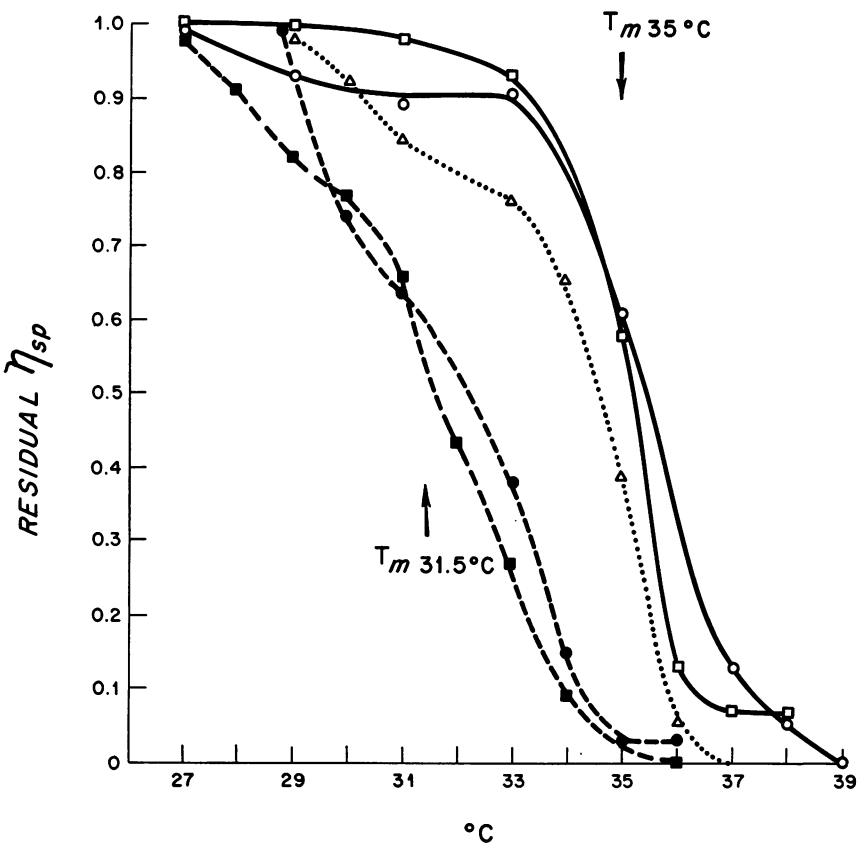

FIGURE 7 Effect of trypsin on residual specific viscosity (η_{sp}) following reaction of collagen in solution at 27°C with synovial collagenase. See text for details. Open circles, squares, and triangles are the curves obtained in the absence of trypsin and closed circles and squares in the presence of 100 μg crystalline trypsin.

precipitates corresponding to the 3/4 and 1/4 length collagen fragments (TC^A and TC^B) described by Gross and Nagai (16) for the tadpole collagenase. In Fig. 8 only the larger fragment (TC^A) is shown in comparison with undigested collagen (TC). Among the segment long spacings prepared from 27°C reaction mixtures the smaller fragments (TC^B) were not found but electrophoresis in polyacrylamide gels of both 20° and 27°C products after thermal denaturation produced bands corresponding to TC^A and TC^B (6). No other bands were seen.

Radioactive products of enzymatic breakdown of ^{14}C-labeled collagen in solution at 27°C, when subjected to gel filtration on Sephadex G-75, emerged in the void volume, consistent with the other observations that they represented large (1/4 and 3/4) fragments of the collagen molecule. Products of enzymatic digestion of labeled reconstituted collagen fibrils at 37°, however, emerged between the cytochrome *c* and ^3H-proline markers. Based on the pattern of elution from these columns and columns of Bio-Gel P-10, their molecular weight was estimated to be in the region 5000–10,000 (6). To determine whether or not the further degradation of the 27°C products to smaller polypeptides was inhibited by EDTA, we incubated ^{14}C-labeled collagen in solution with enzyme in a viscometer at 27°C until the reaction was complete. EDTA was then added and the temperature raised to 37°C for 1 hr. As shown in Fig. 9, *A*, on subsequent gel filtration on Sephadex G-75 the products emerged in the void volume. This was in contrast to the elution pattern of the products resulting from the reaction of gelatin with enzyme for 1 hr at 37°C in the absence of EDTA (Fig. 9, *B*) where the products were retarded on the column. Since the products of the reaction between collagen and enzyme at 27°C are completely denatured at 37°C these observations suggested that, in addition to inhibiting the initial enzymatic cleavage of the collagen molecule, EDTA also prevented the further breakdown of the large fragments to smaller polypeptides.

In view of the difference in the products of reaction of synovial enzyme and collagen in solution at various temperatures, the effect of temperature on the action of the enzyme on collagen *fibrils* was also investigated. This question is an important one since it has not been shown that any collagen

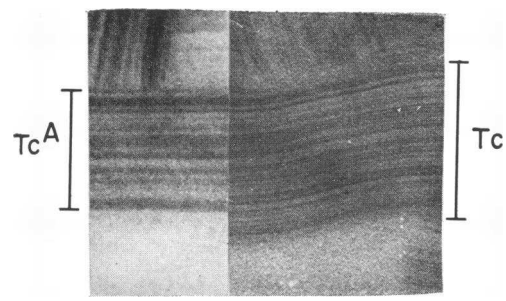

FIGURE 8 Electronmicrographs of the segment long spacings (SLS) of the larger fragment (TC^A) produced by the action of synovial collagenase compared with the undigested collagen SLS (TC). The length of the TC is approximately 3000 A. The particular banding pattern of the asymmetric molecule permits the identification of an approximate point of cleavage of the molecule (16).

is soluble at 37°C in physiological buffers. Therefore, ^{14}C-labeled reconstituted collagen fibrils were prepared by incubating labeled collagen in solution at 37°C for 72 hr, and then reacted with the same quantity of enzyme at temperatures varying from 20°–37°C. Collagenolytic activity was assayed, as previously, by measuring the radioactivity released into the supernatant after centrifugation. The results, shown in Table II, indicate that trivial collagenolytic activity occurred at 20°, 27°, and 30°C compared to the action at 37°C. The rate of dissolution of fibrils increased approximately 5-fold between 20° and 30°C, whereas the rate of dissolution increased over 18-fold between 27° and 37°C. It is also shown in Table II that the low rate of dissolution of the collagen fibrils at 20°C was not due to either inactivation of the enzyme or failure to bind to the collagen fibrils at this temperature. Furthermore, the dissolution of the fibrils which occurred at 37°C after preincubation at 20°C, was not due to a falling apart of fibrils enzymatically cleaved at the lower temperature, since subsequent incubation at 37°C in the presence of EDTA for 2–24 hr failed to release into solution more than 2% of the total radioactivity of the gel.

The effect of synovial enzyme on native collagen *fibers* was studied using guinea pig skin or rat tail tendon as substrate. Fragments of ^{14}C-labeled skin remaining after extraction of salt-soluble collagen were gently agitated for 24 hr in 0.1 M Tris-HCl, pH 7.6, and $CaCl_2$, 0.002 M, at 37°C

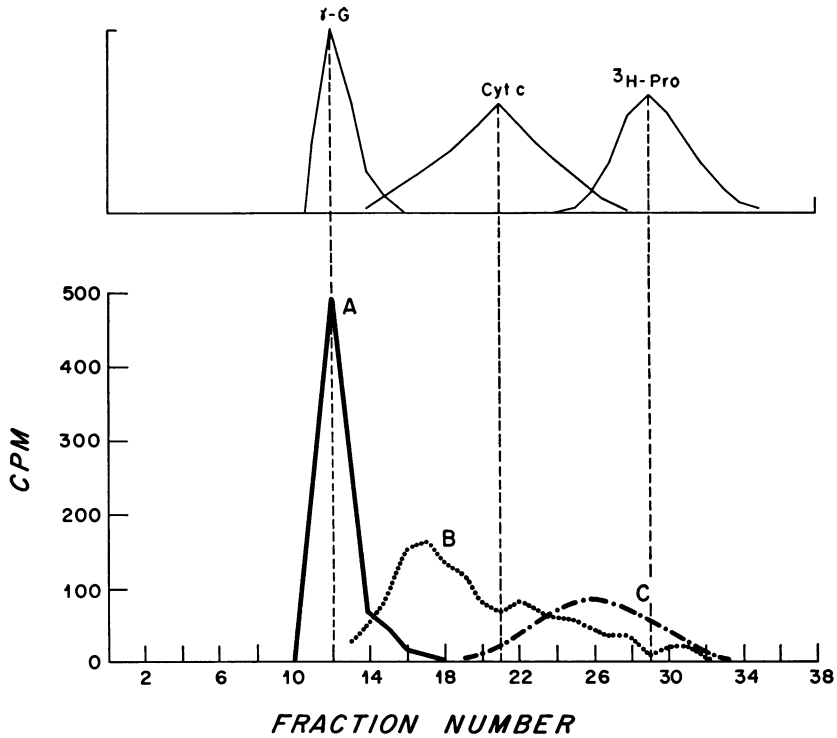

FIGURE 9 Gel filtration on columns of Sephadex G-75 of the products of reaction of synovial collagenase and ^{14}C-glycine–labeled collagen. Initial collagen substrate contained approximately 16,000 cpm. Human gammaglobulin (γ-G), cytochrome *c* (Cyt c), and ^{3}H-proline (^{3}H-Pro) were added as markers. Curve *A* shows the elution pattern for collagen incubated with synovial collagenase at 27°C until the specific viscosity was reduced to about 49% of the initial value and remained at that level. EDTA was then added to final concentration of 0.0025 M and the temperature raised to 37°C for 1 hr. After heat denaturation, the radioactivity was eluted in the void volume of the column. A similar pattern was seen when collagen was heat denatured to gelatin in the absence of collagenase. In curve *B*, is shown the elution pattern for gelatin incubated with enzyme at 37°C for 1 hr in the absence of EDTA. In curve *C* the gelatin was incubated with enzyme at 37°C for 24 hr in the absence of EDTA.

in the presence of enzyme. After centrifugation, radioactivity and total hydroxyproline were measured in the supernatant fluid. The results presented in Table III indicate that exposure to enzyme resulted in release of radioactivity and hydroxyproline into the solution. In other experiments, not shown, rat tail tendons, suspended by a glass weight in buffer and incubated at 37°C, were digested to the point of rupture in the presence but not in the absence of the enzyme.

The synovial enzyme is inhibited when incubated in the presence of serum, both from normal subjects or from patients with rheumatoid arthritis (Fig. 10). Inhibition was demonstrated with dilutions of serum as high as 1 : 1000.

DISCUSSION

The rapid turnover of collagen which occurs during remodelling of connective tissue at periods of growth, injury, and repair, and the much slower process of collagen metabolism which continues throughout the life of the adult animal demand the action of a mechanism capable of breaking down collagen fibrils under physiological conditions of pH and temperature. Before the work of Gross and his colleagues (3, 4, 16) on the metamorphosing tadpole, attempts to identify a specific animal collagenase operative under these conditions had been uniformly unsuccessful. A variety of preparations having some collagenolytic activity had

TABLE II

Effect of Synovial Enzyme on Dissolution of Collagen Fibrils at Different Temperatures

Experiment	Temperature	Enzyme	Incubation time	cpm released	cpm released/hr
	°C		*hr*		
1	20	0	24.0	323	—
	20	+	24.0	432	4.5
2	20	0	22.5	342	—
	20	+	22.5	434	4.1
	28	0	21.5	308	—
	28	+	21.5	515	16.5
	37	0	6.0	254	—
	37	+	6.0	947	115.0
3	20	0	28.0	267	—
	20	+	28.0	453	6.6
	27	0	26.5	264	—
	27	+	26.5	640	14.0
	30	0	18.0	250	—
	30	+	18.0	846	34.0
	37	0	5.0	184	—
	37	+	5.0	1478	259.0

Different enzyme preparations were used in each experiment. Microgels contained approximately 2,000 cpm of ^{14}C-glycine–labeled collagen, incubated at pH 7.4. Values for cpm released per hr are corrected for controls incubated in the absence of enzyme. When the supernatant from the tubes incubated for 28 hr at 20°C (experiment 3) were incubated with fresh labeled gels at 37°C for an additional 5 hr 1525 cpm were released. When the pellets from the same tubes were incubated with buffer without additional enzyme for a further 5 hr at 37°C, 575 cpm were released.

been described (1, 2, 18) but their action on collagen was trivial at neutral pH and their mode of attack on the collagen molecule was largely unexplored. A major advance in this field came with the demonstration by Gross and Lapiere (3) that certain tadpole tissues grown in culture would produce lysis of the collagen gels on which they were cultivated. These workers went on to identify a specific collagenase in the tissue culture medium capable of degrading native collagen at neutral pH and physiological temperatures. Thus, it was established that although an enzyme having collagenolytic properties might not be extractable directly from tissue, it could be demonstrated and isolated using tissue culture techniques.

Chronic rheumatoid arthritis is frequently associated with progressive destruction of collagenous articular and juxta-articular structures such as cartilage, bone, and tendon. Extracts of synovial

TABLE III

Collagenolytic Action of Synovial Enzyme on the Collagenous Residue of Guinea Pig Skin

Tube No.		Wet wt of skin fragments	Radioactivity released	Hydroxyproline released
		mg	*cpm*	*μg*
1	Control	19.2	127	<3
2	Control	17.3	173	<3
3	Control	20.3	120	<3
4	Enzyme	20.6	633	111
5	Enzyme	14.7	556	95
6	Enzyme	13.1	398	120

Incubation was at 37°C, in buffer (Tris-HCl, 0.1 M, pH 7.6; $CaCl_2$, 0.002 M) for 16 hr. The supernatant was then decanted, centrifuged at 20,000 *g* for 20 min, and an aliquot portion assayed for radioactivity and hydroxyproline (11).

tissue from patients with rheumatoid arthritis have been shown to contain enzymatic activity capable of degrading chondromucoprotein over a wide range of pH (19). More recently, cultured rheumatoid synovial cells have been shown to alter the matrix of articular cartilage possibly by proteo-

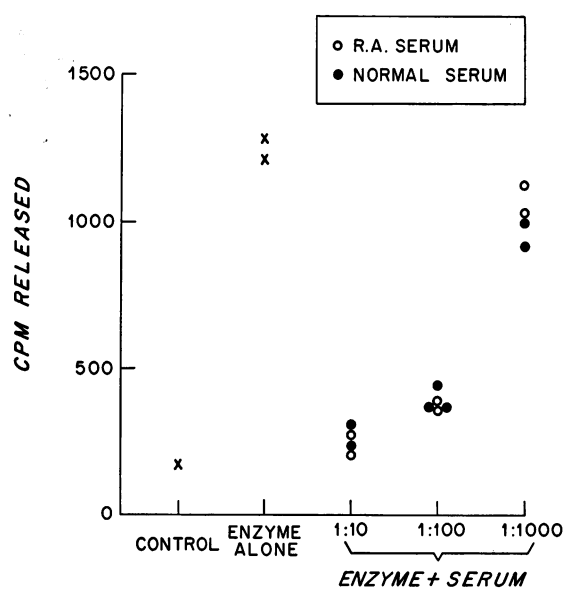

FIGURE 10 Effect of added human serum on the activity of synovial collagenase (microgel assay). On the abscissa are shown the various dilutions of added sera from patients with rheumatoid arthritis (R. A.) and sera from normal subjects. The solubilization of collagen fibrils (cpm released) is depicted in the ordinate. Each tube contained approximately 2000 cpm of ^{14}C-glycine–labeled collagen. Incubations were at 37°C for 3 hr.

lytic cleavage of the protein of the protein polysaccharide (20). However, previous attempts to identify a collagenolytic substance in synovial fluid or in extracts of synovial tissue have failed (21). Using the tissue culture technique, however, a specific collagenase can be identified which, in its action on the collagen molecule, resembles in many respects the amphibian enzyme.

Inability to demonstrate the enzyme in culture media during the first 3 or 4 days of cultivation in vitro might be explained in several ways. During the initial period inhibitory substances such as those found in serum may be present, or alternatively, synthesis of the enzyme may begin only after the first few days in culture and the time course of collagenolytic activity of the medium reflect the true rate of synthesis and release of the enzyme by the surviving tissue. Some preliminary observations (22) suggest that inhibitors are present in the medium during the first few days, but whether these are synthesized in vitro by the tissue or are present within the tissue and are simply washed out during the first few days in culture is as yet unsettled. Although it is possible that the synovial collagenase could be inactivated by a nonspecific proteolytic enzyme, such as trypsin, such a mechanism is unlikely to account for the failure to detect collagenolytic activity in the medium in the first few days of culture since caseinolytic activity was absent at this stage. In those instances where nonspecific proteolytic activity was assayed on daily media, the low levels of caseinolytic activity detected paralleled changes in collagenase activity. It is possible that, like the amphibian enzyme, the synovial collagenolytic enzyme itself has minor nonspecific proteolytic properties, although purification of the enzyme will be needed to establish this point. The lack of activity of the synovial collagenase at pH 5 distinguishes this enzyme from the usual lysosomal enzymes, although proteases active at neutral pH have been localized in some instances to lysosomal granules (23).

The mode of attack of the synovial enzyme on collagen in solution at low temperatures is similar to that of the tadpole collagenase. (Both enzymes attack the undenatured molecule in a susceptible region. Whether or not their action is identical will have to await the demonstration of the extact peptide bond cleaved and the amino terminal groups released.) In the body, however, the natural substrate for the enzyme is presumably collagen in fibrillar form. Exposure of reconstituted collagen fibrils to the enzyme in vitro results in products of low molecular weight (5000–10,000), but this reaction occurs rapidly only at 37°C. At temperatures of 30°C and below, collagen *fibrils* are relatively resistant to the action of the enzyme although undenatured collagen *molecules* in solution are readily attacked. Since the melting temperature of the 3/4 and 1/4 length fragments is close to 35°C, it may be postulated that the initial cleavage of superficial collagen molecules on a fibril at 37°C results in progressive uncoiling of the resulting molecular fragments from the surface of the fibrils into solution. Here they are degraded to smaller polypeptides while further molecules on the fibril surface become exposed to the initial enzymatic attack. At temperatures below the melting point of the large fragments the intermolecular forces binding superficial molecules to the fibril exceed any tendency of the attached fragments to uncoil and the enzyme thus has a limited action. In the presence of trypsin, the midpoint melting temperature (T_m) of the large fragments is reduced, and although the experiments on EDTA inhibition suggest that the synovial enzyme can itself complete the breakdown of the 3/4 and 1/4 length fragments, nonspecific proteases may also participate in this process in the body.

Native collagen fibers in skin and tendon are clearly susceptible to the action of synovial collagenase and elaboration of this enzyme by inflamed or proliferating synovium in rheumatoid arthritis might participate in the destruction of some collagenous structures within and around the joints. The failure to extract collagenase directly from tissues which could be shown to produce the enzyme in culture was discussed by Lapiere and Gross (4). Their suggestion was that synthesis of the enzyme might be a very closely regulated process with minimal storage of active enzyme in the tissue. During tadpole metamorphosis collagenase-producing cells were envisaged as "carrying the enzyme to the substrate" when they invaded collagenous structures. The frequently observed phenomenon that intimate contact between proliferating synovium and bone or cartilage accompanies the damage to these structures in rheumatoid arthritis is in accord with

this interpretation of the mechanism of collagen resorption. The precise cell of origin of the enzyme in our organ cultures of rheumatoid tissue is not yet determined. It seems unlikely that the characteristic inflammatory round cells were involved since histological examination showed that they did not survive in the tissue during culture and few were evident at the time of maximal enzyme yield. Polymorphonuclear leukocytes were usually sparse in the tissue when it was first planted, as is characteristic of rheumatoid arthritis, and were present in even smaller numbers after several days of culture. It is therefore unlikely that enzymes such as those found in extracts of human polymorphonuclear leukocytes (24) would be responsible for the activity observed in these studies. Cultivated connective tissue cells derived from normal synovial tissue have been shown to be capable of synthesizing collagen (25). It remains to be determined whether or not, at other times and under different conditions, the same cells would produce collagenase. So far samples of synovium from patients with degenerative or traumatic arthritis have produced no detectable lysis of collagen gels in culture (26). However, mass cultures of synovium from the hip of a single patient with aseptic necrosis have yielded enough enzyme to characterize the reaction products which were similar to those resulting from the action of the rheumatoid collagenase.[1] Gel lysis was also found in needle biopsy samples of synovium from some patients with inflammatory joint disease other than rheumatoid arthritis (26,[1]).

We have shown that serum proteins inhibit the action of synovial collagenase in vitro. Normal and pathological synovial fluids contain serum proteins in concentrations in excess of 4–5%. The question may therefore be raised how the synovial enzyme could produce changes in the rheumatoid joint in vivo. Preliminary observations in this laboratory [2] have revealed that there is variable inhibition of synovial collagenase by all synovial fluids. However, some synovial fluids from patients with rheumatoid arthritis and other inflammatory joint disease show significantly less apparent inhibition than fluids from patients with degenerative or traumatic joint disease.

A number of human tissues have now been shown to possess collagenolytic activity using tissue culture techniques (27–31) although characterization of the products is incomplete except in the case of the enzyme derived from skin (28), in which the products are similar to those described here. The in vivo mechanism governing the synthesis and release of enzymes responsible for collagen degradation are as yet largely unexplored.

[1] Harris, E. D., Jr., G. L. Cohen, and S. M. Krane. 1968. Synovial collagenase: its presence in culture from joint disease of diverse etiology. Submitted for publication.

[2] Harris, E. D., Jr., and S. M. Krane. Unpublished observations.

ACKNOWLEDGMENTS

We are gratetful to Mrs. Kathleen Grant and Mrs. Eleanor Pyle for technical assistance, to Doctors Edward D. Harris, Jr. and Jerome Gross for helpful advice, and to the orthopedic surgeons who provided us with synovium, particularly Doctors William N. Jones, Theodore Potter, and Roderick H. Turner. This work was supported by Grants AM 3564 and AM 5142 and Training Grant AM 5067 of the U. S. Public Health Service.

REFERENCES

1. Frankland, D. M., and C. H. Wynn. 1962. The degradation of acid soluble collagen by rat liver preparations. *Biochem. J.* **85**: 276.
2. Houck, J. C., and Y. M. Patel. 1962. The collagenolytic activity of pancreas. *Ann. N. Y. Acad. Sci.* **93**: 331.
3. Gross, J., and C. M. Lapiere. 1962. Collagenolytic activity in amphibian tissues. A tissue culture assay. *Proc. Natl. Acad. Sci. U. S.* **48**: 1014.
4. Lapiere, C. H., and J. Gross. 1963. Animal collagenase and collagen metabolism, *In* Mechanisms of Hard Tissue Destruction. R. F. Sognnaes, editor. AAAS., Washington, D. C. 663.
5. Jeffrey, J. J., and J. Gross. 1967. Isolation and characterization of a mammalian collagenolytic enzyme. *Federation Proc.* **26**: 670. (Abstr.)
6. Evanson, J. M., J. J. Jeffrey, and S. M. Krane. 1967. Human collagenase: identification and characterization of an enzyme from rheumatoid synovium in culture. *Science.* **158**: 499.
7. Gross, J. 1958. Studies on the formation of collagen. *J. Exptl. Med.* **107**: 247.
8. Committee of the American Rheumatism Association. 1959. 1958 revision of diagnostic criteria for rheumatoid arthritis. *Arthritis Rheumat.* **2**: 16.
9. Nagai, Y., C. M. Lapiere, and J. Gross. 1966. Tadpole collagenase. Preparation and purification. *Biochemistry.* **5**: 3123.
10. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**: 279.

11. Prockop, D. J., and S. Udenfriend. 1960. A specific method for the analysis of hydroxyproline in tissues and urine. *Anal. Biochem.* **1**: 228.
12. Bergman, I., and R. Loxley. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. *Anal. Chem.* **35**: 1961.
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265.
14. Seifter, S., P. M. Gallop, L. Klein, and E. Meilman. 1959. Studies on collagen. II. Properties of purified collagenase and its inhibition. *J. Biol. Chem.* **234**: 285.
15. Nagai, Y., J. Gross, and K. A. Piez. 1964. Disc electrophoresis of collagen components. *Ann. N. Y. Acad. Sci.* **121**: 494.
16. Gross, J., and Y. Nagai. 1965. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. *Proc. Natl. Acad. Sci. U. S.* **54**: 1197.
17. Kunitz, M. 1947. Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* **30**: 291.
18. Woessner, J. F. 1962. Catabolism of collagen and noncollagen protein in the rat uterus during postpartum involution. *Biochem. J.* **83**: 304.
19. Ziff, M., H. J. Gribetz, and J. Lospalluto. 1960. Effect of leukocyte and synovial membrane extracts on cartilage mucoprotein. *J. Clin. Invest.* **39**: 405.
20. Hamerman, D., R. Janis, and C. Smith. 1967. Cartilage matrix depletion by rheumatoid synovial cells in tissue culture. *J. Exptl. Med.* **126**: 1005.
21. Dresner, E., A. Kang, and P. Trombly. 1962. The differential hydrolysis of soluble and insoluble collagens by collagenolytic enzyme systems. *Arthritis Rheumat.* **5**: 291. (Abstr.)
22. Harris, E. D., Jr., J. M. Evanson, and S. M. Krane. 1968. Stimulation by colchicine of collagenase production by rheumatoid synovium in culture. *J. Clin. Invest.* **47**: 45 a (Abstr.)
23. Weissmann, G., and I. Spilberg. 1968. Breakdown of cartilage proteinpolysaccharides by lysosomes. *Arthritis Rheumat.* **11**: 162.
24. Lazarus, G. S., R. S. Brown, J. R. Daniels, and H. M. Fullmer. 1968. Human granulocyte collagenase. *Science.* **159**: 1483.
25. Castor, C. W., and K. D. Muirden. 1964. Collagen formation in monolayer cultures of human fibroblasts. *Lab. Invest.* **13**: 560.
26. Harris, E. D., Jr., G. L. Cohen, and S. M. Krane. 1968. Collagenase activity in tissue cultures of needle biopsies of rheumatoid and non-rheumatoid synovium. *Arthritis Rheumat.* **11**: 486. (Abstr.)
27. Grillo, H. C., and J. Gross. 1967. Collagenolytic activity during mammalian wound repair. *Develop. Biol.* **15**: 300.
28. Eisen, A. Z., J. J. Jeffrey, and J. Gross. 1958. Human skin collagenase. Isolation and mechanism of attack on the collagen molecule. *Biochim. Biophys. Acta.* **151**: 637.
29. Fullmer, H. M., G. Lazarus, W. A. Gibson, A. C. Stam, and C. Link. 1967. Collagenolytic activity of the skin associated with neuromuscular diseases including amyotrophic lateral sclerosis. *Lancet.* **1**: 1007.
30. Fullmer, H. M., and W. Gibson. 1966. Collagenolytic activity in gingivae of man. *Nature.* **209**: 728.
31. Riley, W. B., Jr., and E. E. Peacock, Jr. 1967. Identification, distribution and significance of a collagenolytic enzyme in human tissue. *Proc. Soc. Exptl. Biol. Med.* **124**: 207.