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AN ENDOPEPTIDASE FROM RHEUMATOID SYNOVIAL TISSUE CULTURE

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SUMMARY

1. An endopeptidase, capable of degrading gelatin to small fragments, has been found in the medium from cultures of rheumatoid synovium. This peptidase, which does not degrade undenatured collagen, has numerous characteristics in common with the rheumatoid synovial collagenase: It is inhibited by EDTA, by serum proteins and by sulfhydryl compounds such as cysteine; it has a broad pH optimum in the neutral range; its synthesis and release does not seem to be stimulated by addition of sucrose to the culture media.

2. The protease has a mol. wt. approximately twice that of the collagenase, and can hydrolyze a chromophore-peptide (Pbz–L-Pro–Leu–Gly–Pro–D-Arg) synthesized as a specific substrate for bacterial collagenase. Using a sensitive assay for neutral protease activity with ^{14}C-labeled gelatin as substrate, the synovial collagenase has a limited capacity to degrade gelatin peptides to small fragments ($<$ 5000 mol. wt.).

3. These data suggest that collagen may be degraded *in vivo*, extracellularly, by at least two enzymes—a collagenase to make an initial cleavage through the collagen molecule and a protease to degrade further the gelatin polypeptides resulting from collagenolysis.

INTRODUCTION

A collagenase, active at neutral pH and inhibited by serum and by EDTA, has been identified in cultures of rheumatoid synovial tissue[1–3]. An enzyme with similar properties as well as another collagenase not inhibited by serum proteins has been found in synovial fluid from certain patients with rheumatoid arthritis[4]. Although assays of synovial tissue obtained by closed needle biopsy have indicated that synovium other than that derived from rheumatoid patients has the capacity to synthesize and release collagenase[5], it seems probable that only in rheumatoid arthritis or in an equally proliferative synovitis is a significant quantity of enzyme produced. Indirect evidence has accumulated[6–8] to suggest that a collagenase may be of primary significance in bringing about the destruction of articular cartilage in rheumatoid arthritis.

During development of a sensitive assay of proteolysis utilizing ^{14}C-labeled gelatin as substrate it was observed that, in addition to the collagenase, protease activity was present in tissue culture media from synovial cultures. This protease did not attack collagen although, similar to the collagenase, it was active at neutral pH and was inhibited by serum and by EDTA. It is the purpose of this report to describe certain properties of this enzyme.

METHODS

[^{14}C]Collagen was purified from skin of guinea pigs (250–300 g), each given an intra-peritoneal injection of a mixture of ^{14}C-labeled amino acids (L-glycine [100 μC]; L-proline [50 μC]; L-leucine [50 μC]; L-amino acid mixture [25 μC] 6 h before sacrifice. Extraction of salt- and acid-soluble fractions of skin were carried out and the collagen was purified by methods reported previously[9,10]. Assays for collagenase activity using both collagen in solution at 20–24° and in fibril form at 37° were performed as described earlier[4]. The method of KUNITZ[11] was used for determination of caseinolytic activity. Methods for culture of synovium in Dulbecco's modified Eagle's medium in the absence of serum, harvesting of media, and subsequent dialysis and concentration of the media have been described[1,2,5]. The procedure was modified, in one experiment, as follows: colchicine (final concentration, 0.25 μM) was added to one set of cultures and sucrose (final concentration, 0.08 M) to another set, while a third set remained as controls. At the end of seven days of culture (media had been harvested daily) the tissue was removed from the flasks, blotted and weighed, and DNA determinations[12] performed on all the tissue from each set of cultures. The media was dialyzed and concentrated before being assayed for several enzyme activities. Colchicine was used because it had been shown to stimulate production and release of collagenase by synovial tissue[13], and sucrose in this concentration has been shown to stimulate production and release of lysosomal proteases from limb rudiments in tissue culture[14]. Assays for collagenase activity using the artificial substrate 4-phenylazobenzyloxycarbonyl (Pbz)–L-Pro–L-Leu–L-Gly–L-Pro–D-Arg were performed as described by WÜNSCH AND HEIDRICH[15], except that solutions were assayed in 0.1 M Tris–HCl (pH 7.6), 5 mM $CaCl_2$. After incubation at 25 or 37° and acidification, the aqueous solutions were extracted with ethyl acetate in which the chromophore dipeptide Pbz–L-Pro–L-Leu is soluble and the absorbance at 320 nm was recorded. Chromatography (thin layer) of the material soluble in ethyl acetate was performed after hydrolysis of the dried samples in 6 M HCl for 18 h at 108°. Cellulose sheets (Eastman 6064) and solvent mixture of *n*-butanol–water–glacial acetic acid (4:1:1, by vol.) were used in an ascending system. Amino acid-containing spots were developed using 0.3% ninhydrin in a solution of *n*-butanol–glacial acetic acid (32:1, by vol.).

Protease assay using [^{14}C]gelatin substrate

[^{14}C]Collagen in a 0.2–0.4% solution in 0.1 M Tris–HCl (pH 7.6), 0.2 M NaCl was heated at 45° for 15 min to form [^{14}C]gelatin. Aliquot portions of 50 μl were placed in disposable, capped polyethylene tubes designed for a Beckman Model 152 microfuge. Aliquot portions (50–100 μl) of sample and 50–100 μl 0.1 M Tris (pH 7.6), 5 mM $CaCl_2$ were added to bring the volume to 200 μl. Control tubes contained 50 μl gelatin solution and 150 μl Tris–$CaCl_2$ buffer. Incubation was carried out at 25 or 37° for

periods of 30 min to 24 h, at which time 100 μl of 45% trichloroacetic acid was added to each tube and the contents mixed. The tubes were placed on ice for 5 min, centrifuged for 4 min in the microfuge, and 150 μl of the supernatant counted in 10 ml Bray's solution[16] in a Packard Model 3320 scintillation spectrometer. Data are expressed in terms of radioactivity or by conversion of radioactivity to weight of gelatin or collagen by determination of the percent of total lysis by the enzyme used.

Chromatographic procedures

Concentrated portions of tissue culture media were dialyzed against 0.1 M Tris–HCl (pH 7.6), 5 mM $CaCl_2$ and passed through a column (2.5 cm × 85 cm) of Bio Gel A - 0.5. Fractions were assayed for collagenase and neutral protease activity. Fractions containing each activity were pooled, concentrated and passed through two columns in tandem (each 0.9 cm × 55 cm) of Sephadex G-150. Samples of gelatin before and after enzymatic digestion were passed through columns of Sephadex G-50 and G-75 equilibrated with 0.5 M $CaCl_2$. The column effluent was monitored by absorbance at 230 nm in a Gilford Model 240 spectrophotometer. Using Bio Gel A-1.5 (200–400 mesh) in 1 M $CaCl_2$, 10 mM Tris–HCl (pH 7.6) as described by PIEZ[17], a column (80 cm × 1.5 cm) was calibrated for molecular weight estimation using α chains and β components of gelatin, the TC^B fragments of collagen produced by synovial collagenase, and smaller peptides prepared by selective cleavage of collagen chains by cyanogen bromide. These peptides (α 1CB2, α 1CB3, and α 1CB4 from chick skin) were kindly provided by Dr. Andrew Kang, and have molecular weights of 3200, 12 900 and 4600, respectively[18].

Miscellaneous

Acrylamide gel disc electrophoresis was performed using a Savant electrophoresis chamber by the method of STARK AND KÜHN[19]. Dithiothreitol was purchased from Calbiochem Corp. Salmon testis DNA and soy bean trypsin inhibitor were obtained from Worthington Biochemical Corporation as was crystalline trypsin treated with L-[1-tosylamido-2-phenyl]ethylchloromethyl ketone to inactivate chymotrypsin. Clostridial collagenase obtained from Worthington Biochemical Corporation was further purified by the methods of SEIFTER *et al.*[20]. Bio Gel A-0.5 and A-1.5 were purchased from Bio-Rad, and Sephadex G-25 and G-50 and G-150 were purchased from Pharmacia Fine Chemicals. Cytochrome *c*, chromatographically pure, was a gift of George McLain. Protein concentration was determined by the method of LOWRY *et al.*[21]. Protease assays at pH 3.5 and 5.0 were performed using hemoglobin substrate (Worthington Biochemical Corp) by the method of ANSON[22]. Isotopically labeled amino acids were purchased from New England Nuclear Corp.

RESULTS

Characteristics of the [^{14}C] gelatin protease (gelatinase) assay

[^{14}C]Collagen with specific activity of greater than 10 000 counts/min per mg was used for the preparation of labeled gelatin. The supernatant solution in samples incubated without enzyme usually contained less than 5% of the total radioactivity, even after incubation for 12 h. Using substrate with a higher specific activity (20 000 counts/min per mg) it was possible to detect lysis of as little as 1 μg of gelatin with an

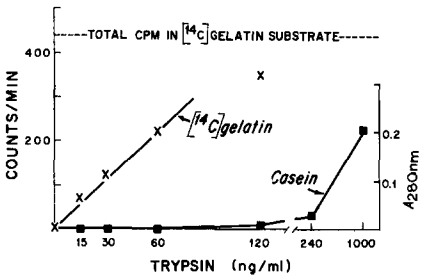


Fig. 1. Comparison of [^{14}C]gelatin and casein as substrates for trypsin. 1 mg of L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone–trypsin was dissolved in 1.0 ml 0.1 M Tris–HCl (pH 7.6), 5 mM $CaCl_2$. Incubation period using gelatin was 2 h at 37°. Incubation period using casein was 8 h at 37°.

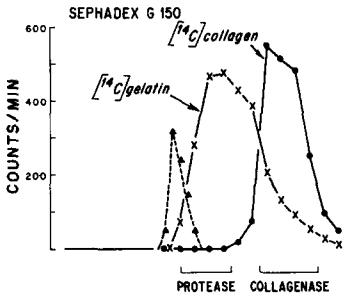


Fig. 2. Elution pattern of crude collagenase preparation from Sephadex G-150. Column sizes and buffers are detailed in the text. The sample was pooled culture media from days 3–6 from a culture of approximately 1.0 g of rheumatoid synovium. Material precipitating in a 20–65% $(NH_4)_2SO_4$ fraction was prepared and dissolved in Tris–$CaCl_2$ and was applied to a column of Bio Gel A-0.5. All fractions were assayed against both gelatin and collagen substrates. Fractions containing protease and collagenase activity were pooled, concentrated, and eluted from G-150 as shown and all fractions were subsequently assayed against both [^{14}C]gelatin and [^{14}C]collagen. Brackets indicate those fractions which were pooled and concentrated for later use. ▲---▲, represents the elution pattern of blue dextran.

incubation period of 12 h. The release of trichloroacetic acid-soluble radioactivity was linear with time until approx. 70% of the total substrate had been degraded. As shown in Fig. 1, this system of assay could detect smaller amounts of proteolytic activity of trypsin than the assay using casein substrate[11].

Separation of collagenolytic and proteolytic activity in rheumatoid synovial culture medium

After passage of concentrated synovial culture media through Bio Gel A-0.5 and assay of each fraction using [^{14}C]collagen and [^{14}C]gelatin as substrates, it was

TABLE I

COMPARISON OF ENZYME ACTION UPON COLLAGEN IN HELICAL AND NON-HELICAL (GELATIN) FORM

Reaction mixtures contained a solution of collagen or gelatin of the same specific activity in 0.1 M Tris–HCl (pH 7.6), 0.2 M NaCl *plus* additional 0.1 M Tris–HCl (pH 7.6), 5 mM $CaCl_2$. Purified collagenase was obtained by $(NH_4)_2SO_4$ precipitation and gel filtration as described in the text. Volumes of crude and purified collagenase were chosen to compare activities in gelatin *versus* collagen after purification of collagenase. Incubation period was 2 h at 37°.

	Trichloroacetic acid supernatant (counts/min)	
	[^{14}C]Gelatin	*[^{14}C]Collagen*
Total	700	700
Control	24	94
Trypsin (25 μg)	700	137
Crude collagenase	689	525
Purified collagenase	78	540

observed that the peaks containing activity against each substrate were not identical. The protease peak emerged closer to the void volume of the column. The two activities could be separated further by chromatography of the pooled fractions containing activity on Sephadex G-150, as shown in Fig. 2. The contents of the tubes containing each activity were then pooled as indicated by the brackets.

The protease assay described above enabled us to use the same protein as substrate either in the undenatured (collagen) or denatured (gelatin) form, both having

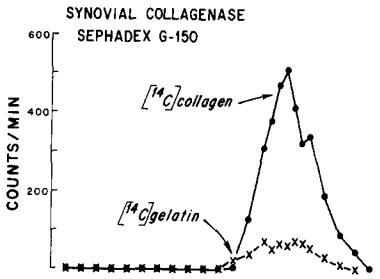


Fig. 3. Comparison of protease and collagenase activity in a purified sample of synovial collagenase reapplied to Sephadex G-150. The total radioactivity assayed either as [^{14}C]gelatin or [^{14}C]-collagen was 700 counts/min.

identical specific activities. In Table I it is demonstrated that synovial collagenase (partially purified by $(NH_4)_2SO_4$ precipitation and subsequent passage through a column of Bio Gel A-0.5 and Sephadex G-150) had less activity against [^{14}C]gelatin than did the crude enzyme, although the activity against collagen of both crude and partially purified preparations used in this experiment were similar. This suggested the possibility that synovial collagenase might have little capacity to cleave gelatin into small fragments. A portion of collagenase purified as described in Table I was re-applied to the column of Sephadex G-150. Fractions collected were assayed using [^{14}C]collagen and [^{14}C]gelatin of identical specific activities (Fig. 3). More radioactivity was solubilized using [^{14}C]collagen than rendered soluble in trichloroacetic acid using [^{14}C]gelatin. We have inferred from these data that the synovial collagenase might have limited specificity and be incapable of degrading gelatin to fragments sufficiently small to be soluble in 15% trichloroacetic acid.

Release of the endopeptidase from synovial cultures in vitro

The data described in Table II indicated that the release of protease activity into synovial culture media at various intervals of culture paralleled the release of collagenase. Only small amounts of both the collagenase and protease were detected during the initial days of tissue culture and these activities peaked on the 3rd and 4th days of culture, respectively. The concentration of sucrose present in culture media in these experiments has been shown by DINGLE *et al.*[14] to stimulate synthesis and release of lysosomal proteases by limb rudiments in tissue culture. In the present system (Table II), sucrose had no stimulatory effect on release of the collagenase as contrasted with the effects of colchicine in low concentrations. Acid protease activity, using denatured hemoglobin as substrate, was not changed in media containing colchicine or sucrose

TABLE II

ENZYME ASSAYS ON MEDIA FROM RHEUMATOID SYNOVIAL CULTURES

Conditions of culture	*Day of culture***	*Collagenase (μg collagen per 100 μg DNA per h)*	*Neutral protease (μg gelatin per 100 μg DNA per h)*	*Acid protease (mequiv tyrosine × 10^{-5} per 100 μg DNA per h)*	
				pH 3.5	*pH 5.0*
(S) Sucrose*	0–1	38	37		
(C) Colchicine**		11	157		
S	1–2	10	132	6.3	1.6
C		144	295	8.1	2.8
S	2–3	19	27		
C		271	458		
S	3–4.5	2	61	14.0	14.8
C		213	620	14.9	14.1
S	4.5–6	2	32	16.5	17.0
C		186	573	10.4	8.8
S	6–7	5	7		
C		174	481		

* Culture media *plus* sucrose, 0.08 M, final concentration.
** Culture media *plus* colchicine, 0.1 μg/ml (0.25 μM), final concentration.
*** Culture media was changed at the end of the interval listed. Conditions of culture and of dialysis, concentration and assay of the media were as described previously[4]. In this particular culture, 30 flasks containing 8, 2 mm × 4 mm synovial explants were used for each of the two groups, S and C.

until days 4.5–6, when tissues containing the sucrose released more enzyme activity.

Characteristics of the synovial protease

The synovial protease was active over a wide range of pH (6.0 to 9.0). At pH

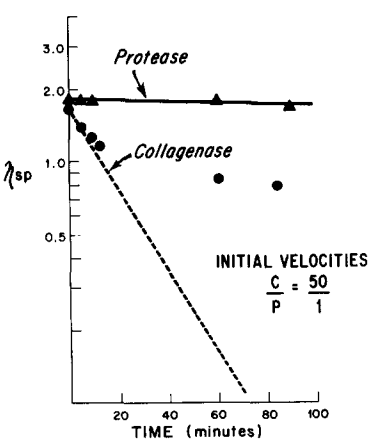


Fig. 4. Collagenolysis by synovial protease and synovial collagenase at 24° in separate viscometers. η_{sp} (specific viscosity) equals 1 — relative viscosity. Mixtures in the viscometers contained 0.4 ml of 0.4% collagen in 0.2 M NaCl, 0.2 ml of 0.4 M NaCl, 0.2 ml 0.1 M Tris–HCl (pH 7.6), 5 mM $CaCl_2$, and 0.2 ml of solution of protease or collagenase. The gelatinase activity of both protease and collagenase preparations used was found previously to be the same.

TABLE III

PROPERTIES OF SYNOVIAL PROTEASE

Inhibitors were either solubilized in Tris-$CaCl_2$ (dithiothreitol, cysteine, soy bean trypsin inhibitor) or added in volumes of 20 μl or less (EDTA). Incubation period was 2 h at 37°.

	Trichloroacetic acid supernatant (counts/min)
Protease	168
Protease + EDTA*	35
Protease + dithiothreitol (5 mM)	38
Protease + cysteine (5 mM)	41
Protease + normal human serum (1:10)	42
Protease + soy bean trypsin inhibitor (0.1 mg/ml)	170
Control	32
Trypsin (25 μg)	548
Total	745

* Sufficient quantity of a 0.1 M solution added to bind Ca^{2+} present (assuming[Ca^{2+}] = 5 mM).

5.2 only 20% of the maximal activity was found and the enzyme was inactive at pH 5.0 or below.

There was negligible activity of the protease against undenatured collagen in solution as assayed by viscometry at 24° (Fig. 4). Comparative rates were approximated by calculating the initial rate of fall in viscosity as a semi-logarithmic function of time. The protease shared several other properties in common with the synovial collagenase including inhibition of its action upon gelatin at pH 7.6 by EDTA, dithiothreitol, cysteine, and a 1:10 dilution of normal human serum (Table III).

p-Chloromercuribenzoate was not inhibitory at a concentration of 5 mM. When [^{14}C]gelatin was incubated with either synovial protease or trypsin in quantities which cleaved the substrate at a similar rate, trypsin was found to be more sensitive to the inhibitory effects of serum than the synovial protease. Whereas the protease was not inhibited by serum in greater than 1:10 dilution, trypsin was inhibited by serum diluted 1:1500.

Although the protease was incapable of degrading native collagen, it did have

TABLE IV

ASSAY OF ENZYME ACTIVITY USING Pbz-PEPTIDE SUBSTRATE

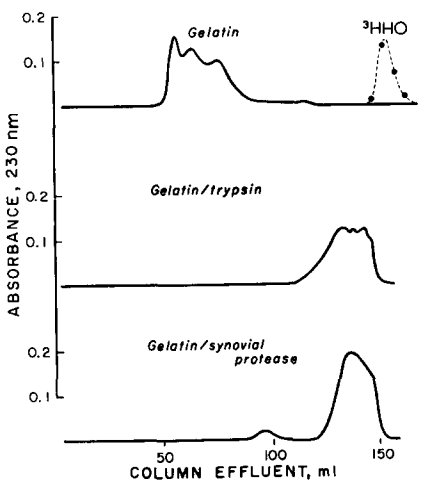
The amount of synovial collagenase used in these assays was sufficient to degrade collagen in solution at a rate more than 50 times that of the synovial protease. Incubation period was 12 h at 37°.

		$A_{320\ nm}$
Bacterial collagenase	0.25 μg	0.023
Bacterial collagenase	0.50 μg	0.049
Bacterial collagenase	1.0 μg	0.123
Synovial protease	50.0 μl	0.435
Synovial collagenase	100.0 μl	0.010
Synovial collagenase	200.0 μl	0.024
Trypsin	50.0 μl	0

the capacity to cleave a compound designed as a specific substrate for bacterial collagenase, Pbz–L-Pro–Leu–Gly–Pro–D-Arg (Pbz-peptide). The results of an illustrative experiment are given in Table IV. Evidence that the Pbz-peptide was cleaved at the Leu–Gly bond was obtained by acid hydrolysis of the peptide extracted into ethyl acetate and identification of proline and leucine on thin-layer chromatograms of the hydrolysate.

As shown in Fig. 4, the synovial protease had negligible proteolytic activity against collagen in solution at 24°. In addition, the synovial protease could not cleave the undenatured TC^A or TC^B fragments produced by synovial collagenase at this temperature. However, when solutions containing TC^A and TC^B were heated to 37°, the TC^A and TC^B were denatured, as shown previously[2], by abrupt decrease in viscosity and optical rotation. Synovial protease was added after denaturation at 37° and incubation continued in the Tris–$CaCl_2$ buffer at pH 7.6 for 48 h. When trichloroacetic acid was added, 95% of the radioactivity of the substrate was soluble and 11% was degraded to fragments small enough to be dialyzable. In contrast, only 3% of the products resulting from the action of trypsin on gelatin were dialyzable although 100% of the radioactivity of the [^{14}C]gelatin was soluble in trichloroacetic acid.

Using a column of agarose it was possible to estimate the molecular size of the fragments produced by the action of the protease upon denatured collagen. ^{14}C-labeled guinea pig collagen (0.5 ml of a 0.3% solution) was incubated with 0.1 ml of the syno-



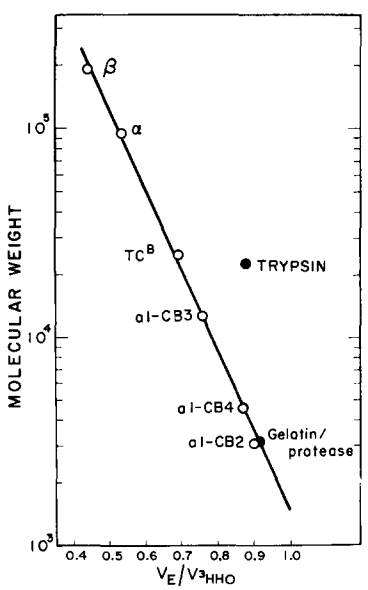


Fig. 5. Patterns of elution of various gelatins from a column of Bio Gel A-1.5 (1.5 cm × 85 cm). The same gelatin was used as substrate in each reaction. Tritiated water (^{3}HHO) was used to mark the bed volume of the column. The 3 peaks in the gelatin sample represent γ, β and α components.

Fig. 6. Log molecular weight *vs.* V_E/V^3_{HHO} of numerous gelatins and one globular protein, trypsin. The ratio for the elution volumn (V_E) of a compound to the volume containing tritiated water V^3_{HHO} is employed since accurate measurement of V_O, the void volume, is difficult using agarose with very high exclusion volumes.

vial protease for 4 h at 37°. 100% of the substrate radioactivity was soluble in trichloroacetic acid, indicating complete breakdown of the gelatin. The remainder of the reaction mixture was applied to a column of Bio Gel A-1.5 (85 cm × 0.5 cm) in 1.0 M $CaCl_2$ and 10 mM Tris–HCl (pH 7.6). As shown in Fig. 5, the reaction products were eluted in a broad peak before the elution volume of tritiated water (^3HHO). Reaction products of trypsin and gelatin were eluted in a similar volume. This same column of agarose was calibrated with numerous gelatin peptides (Fig. 6). The protease apparently has the capacity to degrade gelatin to fragments of a mean molecular size of approx. 3000. It is of interest, as pointed out by PIEZ[17], that a random coil (such as gelatin) will occupy a larger volume than a globular protein of the same molecular weight. This is clearly illustrated by the observation (Fig. 6) that crystalline trypsin (mol. wt. 23 000) was eluted from the column of agarose in the same volume as α1CB4 peptides which have a mol. wt. of 4600. The relatively large volume occupied by the small gelatin polypeptides may account for the observation that, although the reaction products of gelatin and trypsin or synovial protease are completely soluble in 15% trichloroacetic acid, only a small proportion are dialyzable.

It was also observed that in the gelatinase assay described above, gelatin polypeptides remained insoluble in 15% trichloroacetic acid until they were degraded to a size of 5000 mol. wt. or less as determined by gel filtration on calibrated columns of Sephadex G-75. In separate experiments, the TC^B fragments (mol. wt. 24 000[23]) produced by synovial collagenase at 20° and separated on columns of agarose from TC^A were found to be insoluble in 15% trichloroacetic acid. The assay for proteolysis which we have described could not detect lysis of gelatin by an enzyme system capable only of limited hydrolysis to fragments much larger than 5000 mol. wt.

DISCUSSION

Only those enzymes with the capacity to catalyze the cleavage of peptide or other bonds of undenatured collagen should be classified as collagenases. At present, collagenases of human origin derived from skin[24], granulocytes[25], rheumatoid synovial tissue[1,2] and synovial fluid[4] have been isolated and characterized. Each of these enzymes makes its initial attack upon the triple-helical molecule at a similar locus 3/4 the distance from the amino-terminus. The midpoint melting temperature of the fragments produced by synovial collagenase is about 35°. At 37° these fragments would be completely denatured to randomly coiled chains[2]. The synovial protease reported here can degrade these gelatin chains to peptides with a mol. wt. less than 5000, some of which are dialyzable. It is proposed that the protease is responsible for at least part of the subsequent breakdown of the primary products of collagenolysis.

HARTLEY[26] has proposed a classification of the proteolytic enzymes as follows: serine proteinases, thiol proteinases, acid proteinases and metal proteinases. The serine group includes enzymes such as chymotrypsin, trypsin, elastin and plasmin, the active centers of which contain a serine residue that reacts uniquely with organophosphorous compounds; these enzymes are not susceptible to inhibition by EDTA. The thiol proteinases, such as papain, can be stabilized and stored in the presence of reducing agents and metal-chelating agents, and are particularly sensitive to both oxidation and to heavy metals. Acid proteases including pepsin, have peak activity at a pH less than 6.0. In view of the optimum activity at neutral pH, the susceptibility to inhibi-

tion by EDTA and thiol reagents, preliminary classification of the synovial protease would place it in the metal proteinase category.

HARPER AND GROSS[27] have reported separation of collagenase and peptidase activities released by tadpole tissues in culture. Similar to the synovial protease reported here, the tadpole peptidase cleaved the synthetic peptide Pbz–L–Pro–Leu–Gly–Pro–D-Arg which had been designed for assays of collagenase for *C. histolyticum*. It is important to emphasize that the ability of an enzyme to cleave substrates of this composition does not indicate that such an enzyme is a "collagenase" in the sense that it can cleave undenatured collagen. The reports of "collagenases" in mammalian cells[28] and in human serum[29] in which the Pbz-peptide was used as substrate should be re-evaluated in light of the data reported here and by HARPER AND GROSS[27]. However, it is possible that such enzymes acting at neutral pH on the Pbz-peptides are similar to the synovial protease.

Although the differences in apparent mol. wt. and in substrate specificity of the synovial collagenase and protease are important in separating the two enzymatic activities, these enzymes are similar in several respects. Both are active at neutral or slightly alkaline pH, both are inhibited by EDTA and by thiol reagents, neither are inhibited by *p*-chloromercuribenzoate, and the pattern of their appearance in culture media of rheumatoid synovial tissues is similar.

It had been shown previously that the amount of collagenase in the medium from cultures of rheumatoid synovium was markedly increased by the addition of colchicine to the cultures[13]. In the present report parallel increases in the synovial protease were observed in colchicine-containing cultures. The mechanism of this stimulation by colchicine has not yet been elucidated. However, colchicine did not stimulate release of acid proteases and the activities of both collagenase and neutral protease were markedly depressed in cultures incubated with sucrose. In experiments with sucrose added to limb rudiment cultures, DINGLE *et al.*[14] observed stimulation of both production and release of proteases active against denatured hemoglobin at pH 3.5 and 5.0, a phenomenon triggered by endocytosis of sucrose. We observed a small increase in activity of acid protease in media of later days of culture of tissues exposed to sucrose. In view of the opposing effects of sucrose and colchicine on the collagenase and neutral protease on one hand and the acid proteases on the other, we conclude that the mechanism for release of collagenase and the neutral protease cannot be the same as that of the acid proteases.

The assay for protease activity described here is sensitive, reproducible and adaptable for semi-micro analysis. Since the [^{14}C]gelatin is soluble in aqueous solutions of high or low ionic strength over a wide range of pH, this assay may prove useful for determination of cathepsins and other proteases active at acid pH obtained from granule fractions of tissue homogenates. Unlike assays such as those utilizing hide powder linked to a dye as substrate for proteases[30], this method utilizes a soluble substrate. In addition, since larger proteins are precipitated from solution in trichloroacetic acid and differences in quenching from one sample to another are readily corrected for by use of automatic external standardization, enzyme solutions containing pigmented or particulate material can be accurately assayed.

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