Tadpole Collagenase. Preparation and Purification*

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ABSTRACT: The preparation of a collagenolytic enzyme from the medium of tissue cultures of tadpole tail fin and back skin is described. The yield of enzyme from the tissues of thyroxine-stimulated tadpoles was the same as that from nontreated tadpoles and very little activity was produced by the skin of mature frogs. The enzyme was concentrated as much as 300-fold by sequential ammonium sulfate precipitation, gel filtration, starch block electrophoresis, and DEAE-cellulose chromatography. Caseinolytic activity was considerably reduced but was still present at low levels in the final product. The purified enzyme attacks native calf skin collagen in solution at pH 7.6 reducing the viscosity 40–50% at 20° and 75% at 27°, and prevents reconstitution of fibrils in the reaction mixture. Two quantitative assay systems were used, one based on inhibition of fibril formation from collagen solution and the other dependent on release of soluble [^{14}C]glycine-containing peptides from reconstituted collagen fibrils. The purified enzyme has a pH optimum between 8 and 9 and is reversibly inhibited by low concentrations of EDTA and irreversibly inactivated by heating to 60° or by low concentrations of cysteine. It is not affected by diisopropyl fluorophosphate (DFP). The small amount of caseinolytic activity which remains associated with purified collagenase shows the same responses to ethylenediaminetetraacetate, cysteine, and diisopropyl fluorophosphate.

Extracts of tissues in which rapid physiologic resorption of collagen occurs have consistently failed to yield an enzyme which, at neutral pH and physiologic temperature, will attack native collagen (Mandl, 1961; Woessner, 1965; Lapiere and Gross, 1963). However collagenolytic activity has been detected in the medium of cultured tissues such as tadpole tail, gills, and gut, mammalian uterus, bone, and skin wounds (Gross and Lapiere, 1962; Gross *et al.*, 1963; Walker *et al.*, 1964; Grillo and Gross, 1964). This enzyme apparently is synthesized *de novo* from living cells and accumulates in the culture chamber to detectable levels during the period of incubation. No enzyme activity was produced by frozen thawed tissues or by those exposed to puromycin (Gross and Lapiere, 1962; Eisen and Gross, 1965).

The tadpole collagenase has been harvested from the culture medium and purified (Nagai *et al.*, 1963) and its mode of attack on collagen partially characterized. The native molecule is attacked at a single locus and across the three polypeptide chains cleaving off one-quarter length of the molecule at the "B" end. The three-stranded organization and helical conformation remains intact in each fragment (Nagai *et al.*, 1964; Gross and Nagai, 1965; Kang *et al.*, 1966). The physiologic implications of the role of this enzyme in tissue remodeling have been discussed elsewhere (Gross, 1964; Usuku and Gross, 1965; Eisen and Gross, 1965). This paper describes in detail the preparation, purification, assay, and some properties of the tadpole collagenolytic enzyme.

Procedures

Culture Preparation. Bullfrog tadpoles (*Rana catesbiana*) ranging between 3 and 5 in. were obtained commercially from Massachusetts and North Carolina. Animals (50–100) were exposed to a mixture of penicillin (300,000 units), streptomycin (0.25 mg), and chloramphenicol (100 mg) per l. of aquarium water for 24 hr prior to removal of back skin and tail fins under sterile conditions. These tissues were cut into thin strips, *ca.* 1 mm wide, spread upon filter paper disks floating in 10 ml of amphibian Tyrode solution in 105-mm Petri dishes, and cultivated at 37° in a moist atmosphere containing 5% CO_2–95% O_2. The tissues from four animals filled one dish; the tail fin and back skin were kept separate. The culture medium had the following composition: NaCl, 8 g; KCl, 200 mg; $CaCl_2$, 200 mg; $MgCl_2$, 100 mg; NaH_2PO_4, 50 mg; $NaHCO_3$, 1 g; glucose, 2 g; penicillin, 230,000 units, streptomycin, 200 mg; and H_2O, 1500 ml. At 24-hr intervals the fluid in each dish was assayed for collagenolytic activity and examined for bacterial contamination in a phase microscope. In early experiments the media was examined repeatedly for bacterial growth on blood agar plates and in aerobic and anaerobic

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[1] Abbreviations used: DFP, diisopropyl fluorophosphate; TCA, trichloroacetic acid.

broth cultures. Contaminated cultures always revealed large numbers of bacteria upon microscopic examination and were discarded. Moreover, such cultures had markedly diminished collagenolytic activity rather than the reverse; none of the bacteria from contaminated preparations produced collagenolytic activity.

Since enzyme activity appeared to reach a peak after 3 days of incubation the cultures were processed at this time by drawing off the media, centrifuging it at 50,000*g* for 30 min at 5–10°, and passing it through a millipore filter, pore size 0.45 μ. The clear, slightly amber solution was dialyzed in the cold for 48 hr against several changes of distilled water, then lyophilized and stored at −20°.

Because it was suspected that the fall in enzyme activity after reaching the 3-day peak might be due to degradation of collagenase by other proteases, or by denaturation, the technique was modified to include daily harvesting of the medium and replacement with fresh balanced salt solution.

Assay Methods. Two quantitative assay procedures were devised, one based on inhibition of fibril formation (change of opacity assay), the other dependent upon breakdown of radioactive collagen fibrils. Collagen solutions used for the change of opacity assay were prepared by suspending lyophilized purified acid-extracted calf skin collagen in cold phosphate buffer, pH 7.6, Γ/2 0.4, at a concentration of 0.2%. After agitation overnight on a wrist shaker in the cold the viscous solutions were dialyzed against large volumes of Tris 0.05 M, pH 7.6, NaCl, 0.2 or 0.4 M, $CaCl_2$, 3×10^{-3} M, for 24 hr, followed by centrifugation at high speed for 1 hr to remove any undissolved collagen. Solutions of [^{14}C]glycine-labeled guinea pig skin collagen extracted in cold 0.5 M NaCl, pH 7.6, and purified as described elsewhere (Gross and Lapiere, 1962) were handled in the same manner. All collagen solutions were always kept below 5° prior to use.

CHANGE OF OPACITY ASSAY. This method is similar in principle to that devised by Houck and Petal (1962) in which the collagenolytic factor is incubated at 25° with a solution of collagen at neutral pH. The degree of activity is measured by the diminution in opacity of the gel formed on subsequent warming to 37° as compared with that for a control collagen solution. The procedure as described here is quantitative, reproducible, and sensitive; substrate: 1.0 ml of 0.05 M Tris buffer containing 5×10^{-3} M $CaCl_2$, pH 7.5; 0.6 ml of 0.05 M Tris buffer containing 0.4 M NaCl; and 0.2 ml of 0.4% collagen in cold 0.4 M NaCl; enzyme solution: 25–200 μl in 0.5 M Tris (add Tris–NaCl solution to give a final reaction volume of 2.0).

Both substrate and enzyme solution were kept at 0–5° and gently mixed in a flat-bottom microKlett tube in the cold to avoid bubbles. The mixture was incubated 1 hr at 25°, then 25 μl of 0.4 M Na–EDTA, pH 7.6, was added with gentle stirring to stop the reaction. The opacity was measured in a Klett colorimeter using a green filter; the tubes were then transferred to a 37° water bath for 90 min and opacity was read again. Control solutions were of the same total volume, using heat-inactivated enzyme. The opacity decrease was read as the difference in arbitrary Klett units between the control and the experimental tubes. [Opacity units = $\Delta C - \Delta E$, where ΔC is opacity (37°) minus opacity (25°), without active enzyme; ΔE is the same, with active enzyme.]

The unit of enzyme activity is defined as follows: opacity units per milliliter per hour at 25° or opacity units per milligram per hour at 25°, milliliter referring to volume of enzyme solution. Where the coefficient of absorptivity was determined the value of OD_{280} could be used. In these experiments the weight of protein in solution was estimated from the ratios of optical densities at 280 and 260 mμ as described by Warburg and Christian (1941). From the absorption spectrum little nucleic acid was present, even in the crude preparations. The correction factor, *F*, ranged from 0.70 for crude enzyme to 1.0 for the purified preparation. The time refers to the period of incubation at 25° prior to stopping the reaction with EDTA.

The second assay system briefly described elsewhere (Gross and Lapiere, 1962; Lapiere and Gross, 1963) depends on the release of radioactive breakdown products from a substrate of reconstituted [^{14}C]glycine-labeled guinea pig skin collagen fibrils. Gels prepared from 200 μl of collagen solution containing about 400 μg of collagen (800–1000 cpm) in 1-ml plastic centrifuge tubes were incubated for 12 hr at 37° in a water bath. All the gels must be equally opaque, which requires that the tubes be scrupulously cleaned. They were disrupted with a steel needle and reincubated for 1 hr at 37° after adding 200 μl of Tris buffer, 0.1 M, pH 7.5, containing 0.001 M $CaCl_2$. The enzyme solutions (100 μl) to be tested were then added and the tubes were gently agitated and reincubated for various times. The reaction was stopped by adding 100 μl of 0.6 M EDTA and followed by further incubation for an additional hour. The tubes were centrifuged at room temperature for 15 min at 20,000*g* to separate the undissolved collagen. An aliquot part of the solution was assayed for radioactivity in a scintillation or gas-flow counter. The activity was expressed as counts per minute released in the reaction mixture minus the cpm in the supernatant of control gels.

ASSAY FOR NONCOLLAGENOLYTIC PROTEASE ACTIVITY. This method employed casein as a substrate (Kunitz, 1947); reaction mixture: (1) 1 ml of 0.6% casein in 0.05 M Tris–10^{-3} M $CaCl_2$, pH 7.0; (2) 0.2 ml of enzyme. The reaction mixture was incubated for 1 hr at 37° and 2 ml of 0.44 M trichloroacetic acid was added. After 30-min incubation at 37°, the tubes were centrifuged and optical density was measured at 280 mμ.

pH DEPENDENCE. The activity of purified tadpole collagenase as a function of pH was tested in the range 5.6–10.1. In one experiment, three buffers were used to span this range: Tris maleate, pH 7.5–8.0; Tris–HCl from 7.5 to 9.0; and glycine from 8.5 to 10.1. In another, Tris maleate and boric acid–KCl were used with similar results. The radioactive collagen assay was used because the change of opacity method was too sensitive to pH.

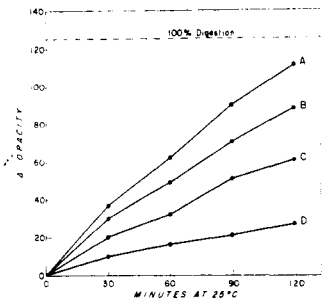

FIGURE 1: Change of opacity assay. Semipurified ($(NH_4)_2SO_4$–G 100) tadpole collagenolytic enzyme. Effect of preincubation time at 25° on collagen gel opacity at 37°. Four different enzyme concentrations: A, 10 μg/ml; B, 7.5 μg/ml; C, 5.0 μg/ml; D, 2.5 μg/ml.

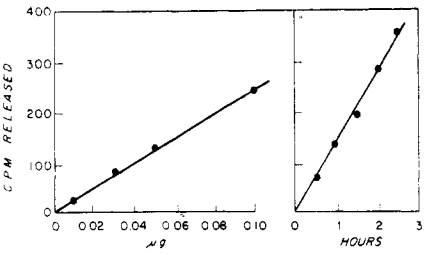

FIGURE 2: Radioactive collagen assay. Purified *Cl. histolyticum* collagenase. Release of radioactivity from [^{14}C]glycine-labeled collagen gel. (A) Degradation of collagen as a function of collagenase after 1 hr of incubation at 37°. (B) Release of radioactive collagen fragments as a function of time of incubation at 37° at an enzyme concentration of 0.05 μg/ml.

Purification of Tadpole Enzyme

Ammonium Sulfate Fractionation. Crude lyophilized enzyme powder (1 g) was dissolved in 50 ml of Tris buffer, 0.05 M, pH 7.6, containing 5×10^{-3} M $CaCl_2$ at 5°; the solution was centrifuged in the cold at 10,000*g* for 10 min and the residue was discarded. To the clear amber supernatant solution in an ice bath solid ammonium sulfate was added to 20% saturation and the preparation was allowed to stand for 30 min. Any precipitate was removed by centrifugation. More ammonium sulfate was added to 50% saturation and the suspension was allowed to stand for 1 hr before removing the precipitate in the centrifuge at 10,000*g* for 10 min. The supernatant solution was discarded. The precipitate was dissolved in 5–10 ml of Tris–$CaCl_2$ buffer, dialyzed against Tris, 0.01 M, pH 7.6, NaCl, 0.2 M, and $CaCl_2$, 5×10^{-3} M, and centrifuged to remove any undissolved residue.

Molecular Sieve Filtration. Gel filtration was accomplished in a water-jacketed column (*ca.* 5–10°) of G 100 or G 200 Sephadex, 4 × 50 cm; Tris buffer (0.01 M), pH 7.6, NaCl (0.2 M), and $CaCl_2$ (5×10^{-3} M) was used for elution. The effluent was monitored at 230 and 280 mμ. Collagenolytic and proteolytic activities were assayed in each tube, and those containing collagenolytic activity were pooled, dialyzed against water, and lyophilized.

Starch Block Electrophoresis. A starch block, 1 × 1.4 × 38 cm, was prepared using a Veronal acetate buffer, pH 8.3, Γ/2 0.057, and 5×10^{-3} M in $CaCl_2$ and precooled at about 5°. Lyophilized enzyme powder (23 mg) was dissolved in 0.6 ml of Veronal acetate buffer and applied to the starch block at the center point. Electrophoresis was carried out in the cold for 28 hr at 400 v and 8 ma. The block was then divided into 1-cm lengths, each of which was eluted with 2 ml of Tris buffer, pH 7.6, 0.05 M. The extracts were clarified in the centrifuge and optical density, collagenolytic activity, and caseinolytic activity were measured.

DEAE-Cellulose Chromatography. The active fractions eluted from the starch block were pooled and dialyzed against 0.005 M Tris, pH 7.5. In a typical experiment 13.5 ml of this solution was chromatographed on a 1 × 4 cm column using stepwise elution from 0.01 to 1 N NaCl. The active fractions were pooled and stored at −20°. Lyophilization at this stage yielded an active product. There was slow loss of activity in the frozen state which seemed to be accelerated at low pH.

Results

Assays of Collagenolytic Activity. The decrement in opacity in the change of opacity procedure, as compared with the control, was directly proportional to collagenolytic activity and increased linearly with time of incubation at 25° (Figure 1). In the presence of a saturating concentration of collagen substrate three different enzyme concentrations produced a constant opacity decrease. The presence of trypsin, chymotrypsin, and extracts of a variety of tissues did not influence the result unless colored substrates or turbidity were present in the reaction mixture; this latter problem could be readily controlled with blanks. Pronase reduced opacity somewhat, but could be distinguished from bacterial or tadpole collagenase in that addition of EDTA at the beginning of the 25° incubation stage did not inhibit the reaction.

In the method which depends upon the release of soluble radioactive fragments from reconstituted fibers, collagen breakdown was linear with enzyme concentration (Figure 2A) and revealed a linear rate of release of radioactivity (Figure 2B). Less than 0.01 μg of purified *Clostridium histolyticum* collagenase (Gallop *et al.*, 1957) can be measured. The kinetics were similar for the tadpole and bacterial enzymes. Most tissue extracts contain proteases capable of releasing 2–8% of the radioactivity in [^{14}C]glycine-labeled collagen. A method was devised (Lapiere and Gross, 1963) for distinguishing between this activity and that of small amounts of bacterial or tad-

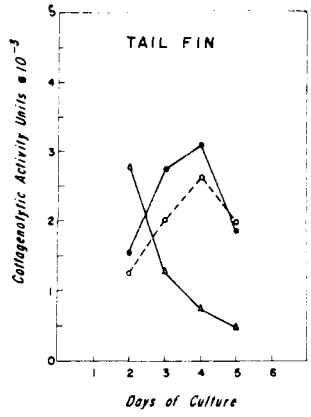

FIGURE 3: Collagenolytic activity in change of opacity units per gram of wet tissue in the media of tail fin cultures as a function of days of incubation at 37°. ○---○, nonmetamorphosing; ●——●, tissues removed for culture 3 days after administering thyroxine to tadpoles; △——△, 5 days after thyroxine administration.

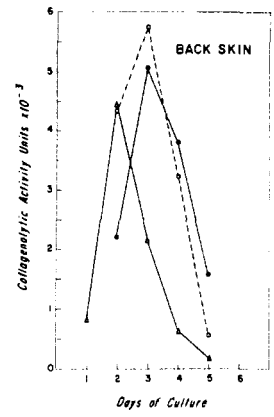

FIGURE 4: Collagenolytic activity in change of opacity units per gram of wet weight of back skin in culture as a function of days of incubation. ○---○, nonmetamorphosing; ●——●, tissues removed for culture 3 days after administering thyroxine to tadpoles; △---△, 5 days after thyroxine administration.

pole collagenase based on the observation (Gallop *et al.*, 1957) that *Clostridium* collagenase can be inhibited by EDTA. The measurement is made by comparing the release of substrate radioactivity by the enzyme in the presence and absence of EDTA in the reaction mixture at 37°, the index being calculated as $(C_0 - C)/C$, where C and C_0 are the released counts per minute in the presence and absence of EDTA, respectively. Trypsin, chymotrypsin, cathepsin C, pronase, papain, and elastase gave values well below 1.0, whereas bacterial and tadpole collagenases had indices well above 1.0 (Lapiere and Gross, 1963). This index is not a quantitative measure of enzyme activity.

The Culture System. Daily measurements were made of collagenolytic activity in each Petri dish over a period of 5 days in a series of cultures of tail fin and back skin. In several experiments post meta-

TABLE I: Enzyme Activity and Recoveries in Stages of Purification.

	Total Protein (mg)	Total Collagenolytic Act.[a]	Sp Collagenolytic Act.[b]	Yield (%)	Total Caseinolytic Act.[c]
1. Crude enzyme prepn (2.23 g) (F = 0.79)	1351	134,200	99.5	100	417
2. 20–50% satd $(NH_4)_2SO_4$ pptn (F = 0.87)	841	114,600	136	85	197
3. Sephadex gel filtration (57 mg) (F = 1.03)	42.3	58,300	1,420	43	95
4. Starch block electrophoresis (F = 1.0)	7.2	50,600	7,020	38	14
5. DEAE-cellulose chromatography (F = 1.08)	1.9	25,400	14,100	19	5.5

[a] Change of opacity units per milligram per hour times the total milligrams of protein in the sample (see pp 10–11). [b] Change of opacity units per milligram per hour. [c] OD_{280} in TCA supernatant/OD_{280} in enzyme sample (see p 13). F is factor based on OD_{280}/OD_{260} for correcting OD_{280} to milligrams of protein (Warburg and Christian (1941)). Total protein (milligram) = $F \times$ total OD_{280} in sample $\times$ $1/d$; d = cell width in centimeters.

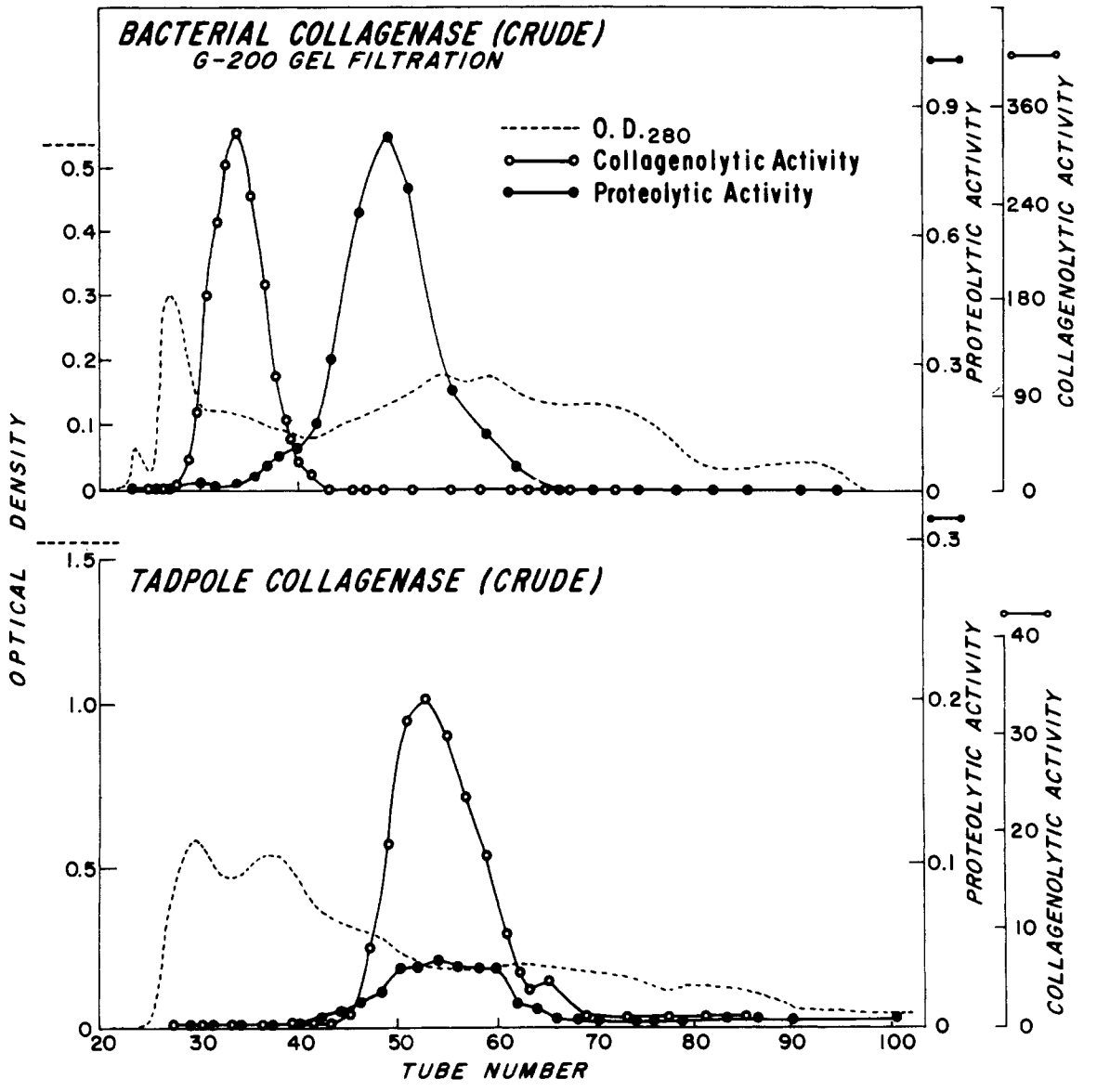

FIGURE 5: Comparison of gel filtration diagrams from crude *Cl. histolyticum* (top) and tadpole (bottom) collagenases. Collagenolytic activity is recorded in change of opacity units and proteolytic activity as the supernatant optical density at 280 mμ in assay using casein as substrate.

morphic frog skin was used. Figures 3 and 4 compare the average daily level of activity in tail fin and back skin and also compare the effect of *in vivo* thyroxine treatment for 3 and 5 days with that for untreated animals. The latter experiment represented an attempt to increase the yield of enzyme.

It is evident that per gram wet weight, back skin produced about twice the collagenolytic activity as did tail fin. This is not surprising, however, in view of the fact that back skin contains about four times the cell concentration as measured by deoxyribonucleic acid (DNA) content (Lapiere and Gross, 1963). Pretreatment of the tadpoles with thyroxine decreased the time prior to the appearance of the enzyme in the culture medium but did not significantly increase the yield; the peak activity appeared earlier but the fall-off also occurred earlier.

In those experiments in which the culture medium was replaced daily, considerable enzyme activity was evident by the second day and in some experiments the cells continued to produce fresh enzyme at relatively high levels for as long as 8 days. Harvesting the medium daily or every 2 days provided large total yields.

Various types of culture media were tried in an effort to improve the yield of enzyme. There was little evi-

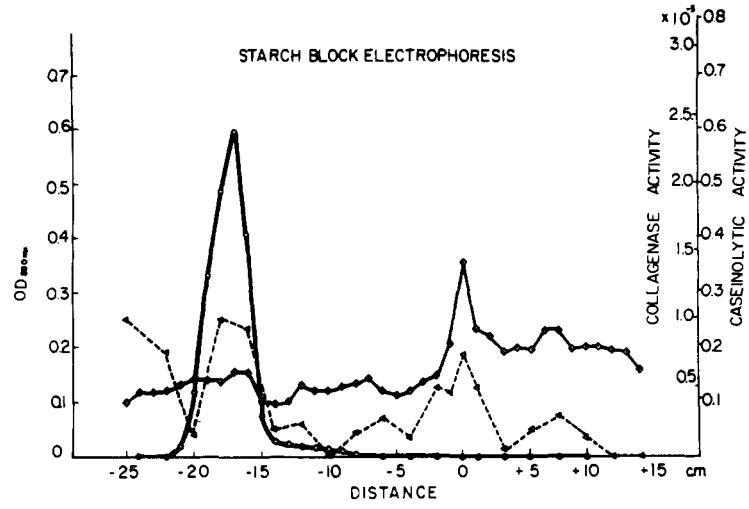

FIGURE 6: Starch block electrophoresis of tadpole collagenolytic enzyme produced by back skin, purified by ammonium sulfate–G 100 filtration treatment. Veronal acetate buffer plus 5×10^{-3} M $CaCl_2$, pH 8.3, $\Gamma/2$ 0.057. Starch block $1 \times 1.4 \times 38$ cm, 400 v, 8 ma, at 4° for 28 hr. After electrophoresis the block was divided into 1-cm lengths, each of which was eluted with 2 ml of 0.05 M Tris buffer, pH 7.6, at 4°. Collagenolytic activity measured in change of opacity units, O——O; △---△, caseinolytic activity; □---□, OD_{280}.

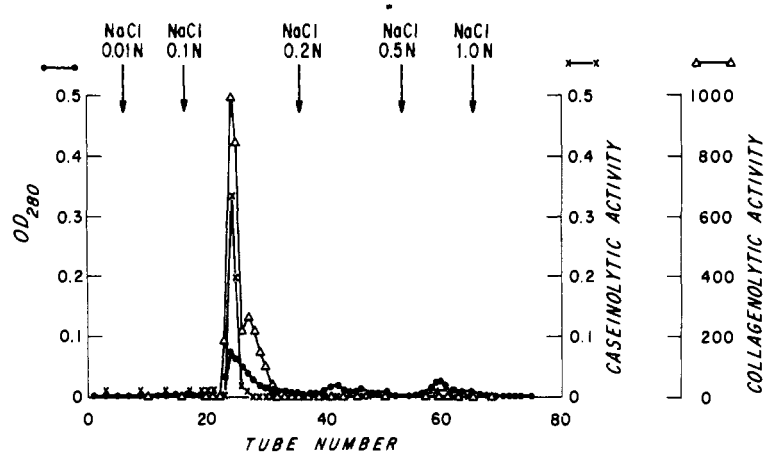

FIGURE 7: Chromatography on DEAE-cellulose (1×4 cm using 0.005 M Tris, pH 7–5, as the initial buffer with a stepwise elution to 1.0 N NaCl. Tadpole collagenolytic enzyme (13.5 ml) from back skin purified by starch block electrophoresis was chromatographed; OD_{280} = 0.158; total change of opacity units, 5870. Collagenolytic activity was measured in change of opacity units per milliliter per hour at 25° (△——△) and caseinolytic activity in OD_{280} units per milliliter per hour at 37° (×———×); OD_{280}, ●———●.

dence to suggest that supplementation of the basic medium with various concentrations of gelatin or collagen had much effect on either the time of appearance or extent of enzyme activity. Bovine serum had a marked inhibitory effect. Addition of thyroxine to cultures of tissue from nonmetamorphosing animals had no influence. Cultures of frog skin failed to yield much active enzyme. The recovery of crude, active powder from the culture medium of back skin or tail fin from 35 tadpoles is about 100 mg, containing variable amounts of activity ranging from 30 to 300 opacity units/mg.

Purification. The recoveries and degree of purification at each stage of fractionation are reported in Table I. The enzyme elutes from the Sephadex column as a discrete peak at a highly reproducible location in the elution diagram (Figure 5, bottom). Most of the material absorbing at 280 mμ was eluted well ahead of the enzyme peak. Although there is a partial separation of nonspecific proteases, a significant amount of ca-

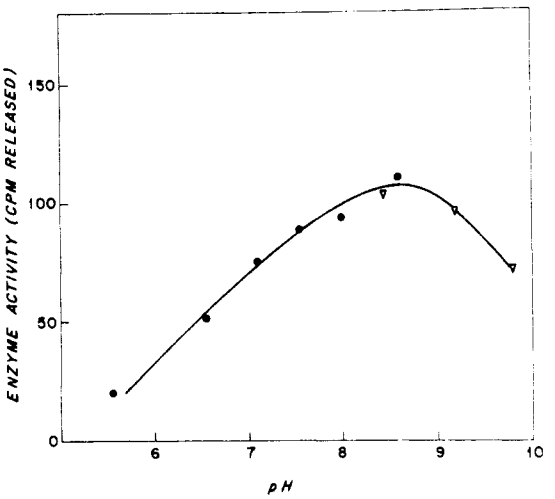

FIGURE 8: Dependence of collagenolytic activity upon pH. Radioactivity assay. Purified back skin collagenolytic enzyme. ●——●, Tris-maleate buffer; △——△, boric acid-KCl-NaOH buffer.

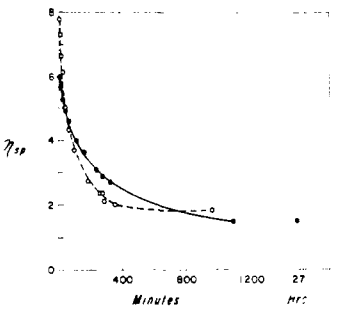

FIGURE 9: Viscosity reducing activity of crude and purified tadpole collagenolytic enzyme. Collagen concentration 0.15% in 0.4 M NaCl plus 0.05 Tris, pH 7.5. Reaction at 27°, ●——●, crude enzyme powder, 20 mg/ml; O---O, purified enzyme, 10 μg/ml.

selinolytic activity remains associated with the collagenolytic enzyme. When one compares the elution pattern of crude *Cl. histolyticum* collagenase (Figure 5, top) with that of tadpole enzyme it is evident that the former leaves the column much earlier. Caseinolytic activity in the two crude enzyme preparations elute in the same volume. When a mixture of the two collagenases was placed on the column a complete separation was obtained.

Electrophoresis on starch block at pH 8.2 (Figure 6) effected a significant increase in specific activity of the collagenolytic enzyme and further reduction of protease activity to $^1/_{25}$ that of trypsin. Collagenase specific activity was increased fivefold with considerable reduction of protease; however the product was contaminated with dissolved starch. In order to eliminate this contaminant the concentrated eluate from the starch block was passed over a short DEAE-cellulose column using batchwise elution at increasing NaCl concentrations (Figure 7). Although a further increment in collagenolytic specific activity was obtained, a 50% loss in enzyme was incurred. Caseinolytic activity again was reduced but not eliminated. In one preparation collagenolytic activity was increased 300-fold by this procedure.

pH Dependence and Ultraviolet Absorption. Crude enzyme gave a typical bell-shaped curve with a broad range of activity between pH 6 and 8 and rapid loss on either side, quite similar to that for *Cl. histolyticum* collagenase. The peak was sharper for purified enzyme, with the maximum occurring between pH 8 and 9 and 70% of peak activity at pH 7 (Figure 8). Purified back skin or tail fin collagenolytic enzyme in solution in 0.005 M Tris buffer yielded a typical protein absorption spectrum the characteristics of which did not change appreciably after the gel filtration step.

Effect of Tadpole Enzyme upon Viscosity. At 27° and pH 7.6 both crude and purified tadpole enzyme in concentrations of 20 mg and 10 μg/ml, respectively, reduced the viscosity of an 0.05% solution of collagen to about 25% of the control level within 10 hr (Figure 9), at which point there was little change upon the addition of fresh enzyme. At 20° the viscosity was reduced to 50–60% of the original value (Gross and Nagai, 1965).

Inhibitors of Collagenolytic and Caseinolytic Activity. Both collagenolytic and caseinolytic activity were destroyed by heating between 50 and 60° for 10 min; there was little loss in activity in 10 min at temperatures below 50°. EDTA in concentrations of 2×10^{-3} M and greater completely inhibited both types of enzyme activity, but they were recovered upon dialysis against low concentrations of calcium chloride. Reduction of pH to 3.5 with acetic acid completely and irreversibly destroyed activity. Cysteine at 5×10^{-3} M completely and irreversibly inhibited both enzyme activities. Diisopropyl fluorophosphate in concentrations of 10^{-2} M had no effect on either enzymatic function.

Discussion

While it has been obvious that some mechanism for the degradation of collagen must be present in animal tissues, the isolation and characterization of the enzyme system has proven most elusive. Collagenolytic activity in extracts from a number of mammalian tissues such as uterus, liver, bone, pancreas, and skin have been reported to occur in the low pH range between 3.5 and 5.8 at mammalian body temperature. However, mammalian collagen in solution at acid pH will undergo progessive denaturation at temperatures above 30° (C. M. Lapiere, unpublished observations), making it readily susceptible to proteolysis. Thus assays of collagen degradation by proteases under these conditions are difficult to interpret.

There are a number of striking differences between tadpole and *Cl. histolyticum* collagenases. The tadpole enzyme seems to be a smaller molecule since it shows greater retardation on a Sephadex column. Perhaps the most radical difference is in their mode of attack on the collagen molecule (Mandl, 1961; Nagai *et al.*, 1964; Gross and Nagai, 1965; Kang *et al.*, 1966). The two enzymes are similar in that they are both inhibited reversibly by chelating agents such as EDTA and irreversibly by cysteine but neither is susceptible to DFP. They have similar pH-dependence characteristics, being most active in the neutral or mildly alkaline range.

In view of the different mode of action (Nagai *et al.*, 1964; Gross and Nagai, 1965; Kang *et al.*, 1966) of the tadpole enzyme on native collagen molecules as compared with that of *Cl. histolyticum* (Mandl, 1961) we suggest that a more inclusive and useful definition of a collagenase might be an enzyme which breaks covalent bonds within the polypeptide backbone of the collagen molecule, causing a change in size, shape, configuration, or stability under conditions which will not in themselves denature the protein. Because the only true collagenase known and characterized has been that from *Cl. histolyticum*, the definition has been highly specialized. Since it is now likely that the animal collagenases differ considerably in their properties and mode of action and may constitute a much larger and diverse group, this suggested broadening of the definition seems justified. Since proteases such as trypsin and chymotrypsin lower the viscosity of collagen solutions slightly and may liberate up to 8% of [^{14}C]glycine-labeled peptide material at 20–27°, we found it necessary to control the assay system by comparing collagenolytic activity with the action of trypsin under identical conditions. Activities in the trypsin range are suspect. However, under certain conditions such as very high enzyme concentration and long periods of incubation in 0.5 M $CaCl_2$, trypsin will attack the main chains of collagen in solution at neutral pH and 20° (Olsen, 1964). No definitions are absolute.

Using a purification sequence involving four different fractionation procedures we have succeeded in eliminating a large proportion of the noncollagenolytic protease activity. However there remains an irreducible small capacity to digest casein which follows the collagenolytic activity and seems quite similar in its sensitivity to EDTA and insensitivity to DFP. It may be that this collagenolytic enzyme also has the ability to cleave certain other proteins.

It is of interest that the output of collagenolytic enzyme by the tissues of thyroxine-stimulated tadpoles undergoing metamorphosis is not much increased above that for tissues from nonmetamorphosing tadpoles. Nor does the addition of thyroxine to the culture accelerate this process. We suggest that isolated tissues when in culture undergo spontaneous metamorphosis and produce enzyme at maximum speed. The observation that frog skin in culture fails to produce much collagenolytic enzyme is consistent with the likelihood that this enzyme is a physiologically significant factor in metamorphosis.

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