# A COLLAGENOLYTIC FACTOR IN RAT BONE PROMOTED BY PARATHYROID EXTRACT*

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The mechanism of collagen resorption in bone requires the presence of an enzyme capable of degrading undenatured collagen under physiologic conditions of pH and temperature. Stern et al (1963) have obtained indirect evidence for collagenolytic action in bone by measuring the release of labeled collagen fragments in the medium of cultures of $H^3$-proline labeled calvarium. They have also shown a two- to threefold increase in activity upon addition of parathyroid extract to the cultures.

Recently Gross and Lapiere (1962) identified and measured a collagenolytic factor produced by cultured tadpole soft tissues and differentiated this activity from that of other proteolytic enzymes (Lapiere and Gross, 1963). These observations depended on the use of a tissue culture system in which the collagenolytic factor accumulated in the medium, revealed itself by lysing a reconstituted, undenatured, $C^{14}$-labeled collagen substrate, and was measured by the radioactivity released.

We report here the demonstration of a diffusible collagenolytic enzyme system in metaphyseal bone of rats treated with parathyroid extract.

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## METHODS

Preparation of Animals -- Sprague-Dawley albino rats 1 to 2 weeks of age received parathyroid extract (Eli Lilly and Co., Indianapolis, Ind.) intraperitoneally in dosages of 10 units per gram. At autopsy each of 6 long bones (humerus, femur and tibia, bilaterally) were excised and the diaphysis at each end transected 1-2 mm away from the epiphyseal plate. Only the osteoclast-rich region of the distal fragments was used for culture.

Preparation of Cultures -- The collagen gels for culture were prepared according to a previously described technique (Gross and Lapiere, 1962), using 0.66 mg of collagen in 1 ml of a modified Eagle protein free medium in Leighton tubes instead of microslide chambers. Several preparations of lyophilized purified $C^{14}$-glycine labeled guinea pig dermal collagen (S.A. 3600 cpm/mg and 6800 cpm/mg) which had been stored at -20.C were used in the present investigation. Twenty mg tissue samples were planted 13 hours after forming the gel at 37° C. Three days later the cultures were centrifuged at 50,000 g for 20 minutes at 26°C. After determining pH, a 200 µl aliquot of supernatant of each culture was counted in 10 ml of Bray's solution for one hour in a automated liquid scintillation spectrometer. The remaining solution was dialyzed against 5 ml of water for 24 hours at 5°C, and the outside fluid dried, redissolved in 200 µl of water and counted.

## RESULTS

The native, or undenatured state of the collagen gels was tested by incubation in quadruplicate with crystallized trypsin (100 µg/ml) substituting for tissue under conditions identical with those for the cultures. Maximum solubilization of 7% of the substrate, above the levels of the blanks, occurred after 3 days of incubation at 37°C.

As shown in Table I, bone fragments from control animals (not hormone treated) degraded a small and relatively constant amount of collagen averaging 13%, about twice the activity of trypsin, whereas there is a five- to tenfold increase in collagenolytic activity in treated rats, also reflected in the increase in dialysable radioactivity.

TABLE I

COLLAGENOLYTIC ACTIVITY MEASURED BY RELEASE OF RADIOACTIVITY FROM $C^{14}$ COLLAGEN GELS AFTER 3 DAYS OF CULTURE

| | | | Increase in CPM over Blank | | | |
|---|---|---|---|---|---|---|
| Culture* | Blank CPM | | Control | | Parathyroid Extract | |
| | Supernate | Dialysate | Supernate | Dialysate | Supernate | Dialysate |
| Exp. I** | | | | | | |
| a | 244 | 13 | 148 | 113 | 1247 | 498 |
| b | 381 | 11 | 210 | 148 | 1001 | 669 |
| a | | | 196 | 138 | 1288 | 550 |
| b | | | 321 | 138 | 1046 | 775 |
| a | | | 168 | 108 | 988 | 482 |
| b | | | 148 | 101 | 932 | 512 |
| a | 301 | 34 | 94 | - | 1045 | 505 |
| b | 263 | 30 | 139 | 31 | 1104 | - |
| a | 282 | 33 | 215 | 78 | 690*** | 414 |
| b | | | 261 | 168 | 551*** | 354 |
| a | | | 192 | 54 | 791*** | 426 |
| Exp. II** | | | | | | |
| a | 325 | | 765 | | 1765 | |
| b | 350 | | 365 | | 2160 | |
| a | 330 | | 405 | | 2535 | |
| b | 395 | | 480 | | 2250 | |
| a | | | 500 | | 1775 | |
| b | | | 500 | | 2350 | |

*Each pair of cultures (a, b) represent tissue samples from two sides of one rat.

**Collagen used in Exp. I contained 2140 cpm/culture and a second preparation used in Exp. II provided 3600 cpm/culture.

***Animals treated with 100 units of hormone as compared with 200 units for all others.

The time dependent action of parathyroid extract in vivo was tested in 26 male 10-day old rats which received 200 U.S.P. units in a single intraperi-

toneal injection and 3 untreated animals serving as controls. The experimental animals were sacrificed at intervals up to 48 hours after hormone administration. Visible lysis of collagen developed first and progressed most rapidly in the cultured metaphyseal fragments from the animals sacrificed 36 hours after receiving hormone (Table II). The 36 hour preparations liberated an average of 52% of the activity originally present in the gel. The degree of collagenolytic response elicited during the 3-day culture of metaphyseal samples obtained from rats which had received 3 successive parathyroid injections at 12 hour intervals (columns a, b and c, Table II) was not greater than that obtained after a single injection (columns d, e, Table II).

TABLE II

IN VIVO EFFECT OF PARATHYROID EXTRACT ON COLLAGENOLYTIC ACTIVITY OF METAPHYSEAL TISSUE WITH TIME AFTER INJECTION.

| | Hours | Increase in CPM over Blank | | | | | pH |
|---|---|---|---|---|---|---|---|
| Experiments | | (a) | (b) | (c) | (d) | (e) | |
| Control | | 249 | 94 | 139 | 175 | 75 | 7.25 |
| Parathyroid Rx | | | | | | | |
| | 4 | | | | 160 | 55 | 7.55 |
| | 8 | | | | 180 | 75 | 7.15 |
| | 12 | | | | 195 | 195 | 7.45 |
| | 18 | 685 | 255 | 221 | 130 | 250 | 7.55 |
| | 24 | 952 | 520 | 315 | 830 | 1030 | 6.50 |
| | 36 | 1643 | 1045 | 1104 | 910 | 1320 | 6.75 |
| | 48 | 1773 | 819 | 893 | 600 | 1080 | 7.60 |

Attempts were made to extract a collagenolytic factor in cold saline homogenates of metaphyseal bone from untreated animals. Supernatant solutions and whole homogenates were placed on bits of moistened filter paper on labeled collagen gels. No increased release of radioactivity over that of the blanks was found after 3 days of incubation. Results with tissue

from hormone treated rats were equivocal. Epiphyseal cartilage and diaphyseal marrow from 36 hour hyperparathyroid animals, degraded about 10% of the original gel while activity of control cartilage and mid-diaphyseal marrow, stomach and liver were negligible.

## DISCUSSION

Until it can be shown that degradation of collagen *in vivo* occurs at low pH we propose that detection of physiologic collagenolytic activity requires the use of undenatured collagen at physiologic temperature and pH. Denaturation of the substrate during preparation and its resulting susceptibility to non specific proteases may be measured by its susceptibility to degradation by trypsin. This enzyme in concentrations of 0.01% causes less than 10% breakdown of native reconstituted fibrils at 37°C and the degradative processes cease in less than 3 hours (Lapiere and Gross, 1963), criteria met in these experiments. Woods and Nichols (1963) report slow continuing degradation of their substrate by trypsin at a rate "less than one-third as rapid as equivalent amounts of collagenase" over a 6 hour period. Also, in contrast with their findings, we were unable to detect collagenolytic activity in control metaphyseal homogenates.

The *in vivo* action of parathyroid extract as compared with controls might be attributed either to an increased number of cells or to an increase in enzyme output per cell. *In vitro* hormone experiments may clarify this point.

The diffusible nature of the collagenolytic factor was demonstrated by the increasing area of visible lysis well beyond the tissue explant. We are attempting to isolate the enzyme system from the culture medium in a manner similar to that accomplished for tadpole tissue cultures (Nagai *et al*, 1963, 1964).

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