Collagenolytic Activity in Mammalian Bone

Abstract. *Collagen from bone was incubated with bone-cell homogenate. At the end of incubation the collagen had been partially broken down to ultrafiltrable units indicating that ferments with collagenolytic activity, which can be released by homogenization, exist in bone cells.*

Although interest in bone resorption has been increasing in recent years, the nature of the processes has remained obscure. While it has been made clear that both the mineral and organic matrix are removed simultaneously or nearly so, the apparent absence of enzymes that can degrade collagen—the major component of the matrix—from mammalian tissues other than pancreas (*1*) complicated the problem. The recent demonstration of collagenolytic activity in tadpole tissue in tissue culture (*2*) encouraged the belief that a similar activity should be demonstrable in mammalian tissue. Bone, undergoing resorption and remodeling, seemed an excellent tissue in which to seek such an activity.

This preliminary report describes experiments which appear to demonstrate a ferment that can break down collagen to ultrafilterable peptides in fresh homogenates of cells isolated from bone.

Substrate for these experiments was prepared by incubating minced rat metaphyseal bone in Krebs-Ringer solution buffered with HCO_3^- and 5 percent CO_2 containing 11.1m*M* glucose and 40 μc of proline uniformly labeled with C^{14} at 37°C for 20 hours. The medium also contained 2000 units of penicillin per milliliter and 2 mg of streptomycin per milliliter to prevent bacterial contamination. After incubation the bone was chilled and washed three times in nonradioactive medium to remove any loose radioactive material. It was then shaken overnight in 0.1*N* NaOH to remove cellular proteins and decalcified by shaking in 10-percent EDTA for 48 hours; the lipids were then removed by shaking in a mixture of ethanol and ether (1 : 1) for 24 hours. All steps were carried out at 0 to 3°C. The collagen so obtained had a specific activity of 3900 count/min per milligram (dry weight). It was stored dry in the cold and ground to a fine suspension immediately before each experiment. The protein isolated from bone by this technique has the amino acid composition of pure collagen (*3*); by chromatography of hydrolysates the radioactivity is entirely in proline and hydroxyproline.

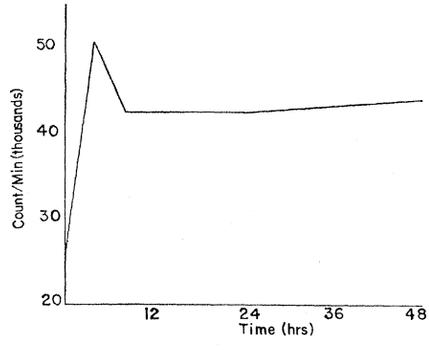

Fig. 1. Specific activity of collagen during 48 hours of incubation of bone chips in "cold" Krebs-Ringer medium. The bone had previously been incubated for 4 hours in medium containing 30 μc of proline-1-C^{14}. The ordinate is counts per minute per milligram of nitrogen.

Histologically intact bone cells were extracted from the metaphyses of 50- to 60-day-old male rats of the Charles River strain by grinding fresh bone in a mortar with abundant isotonic saline solution containing 1.5m*M* Ca and 1.5m*M* P. The cells were removed from the supernatant after sedimentation by centrifugation (*4*). They were suspended in bicarbonate-buffered Krebs-Ringer medium and homogenized in an all glass homogenizer or a Waring blender. All operations were performed at 0° to 3°C in a cold room, and the homogenates of the cells were kept cold until used.

Flasks were prepared which contained 5 mg of collagen, in fine suspension, and 0.5 ml of the cell homogenate buffered at *p*H 7.4. All flasks were incubated at 37°C for 6 hours. Control flasks contained collagen and Krebs-Ringer medium alone. After incubation, the contents of each flask were filtered overnight through cellophane tubing (*5*). This tubing has a pore diameter that retains substances of 20,000 to 30,000 molecular weight or greater. The radioactivity of this ultrafiltrate was estimated by liquid-scintillation counting, after evaporation to dryness and dissolving the dry material in concentrated formic acid.

Table 1 shows the results from a series of experiments. In the presence of the homogenate of bone cells, eight times more collagen substrate was degraded to fragments which were ultrafilterable than when the homogenate was absent. This activity was completely abolished by heating the homogenate to 70°C for 2 minutes. The addition of sufficient EDTA to bind all the divalent cation in the system inhibited the activity 35 percent; maximum activity appeared between *p*H 6 and 7. Comparison with purified bacterial collagenase (Sigma type 1) revealed that the activity of 100 mg of dry homogenate upon the collagen substrate was approximately equivalent to 0.10 μg of dry enzyme.

Table 1. Radioactivity of ultrafiltrate obtained from samples of collagen after incubation with cell homogenate, and alone in Krebs-Ringer medium for 6 hours at 37°C. Total amount of collagen present initially represented 20 to 40,000 count/min. The results are expressed as counts per minute per total sample.

Expt.	Cell homogenate + collagen	Collagen alone
1	4050	720
2	1438	62
3	1429	109
4	1105	67
5	3591	161
6	2063	282
7	1595	81
8	1371	365
9	1450	30
10	1260	435
11	1070	130
Mean	1765	222

The possibility arose that the particulate collagen substrate in these experiments might have been so denatured during preparation that it was susceptible to the action of nonspecific cathepsins. However, electron microscopy of the substrate revealed many small fibers with all the morphologic characteristics of native collagen. Moreover, while the *p*H of the alkali was 12.3 when added to the bone, the buffer action of the bone mineral rapidly lowered the *p*H of the mixture to 7.8. Thus the collagen was not exposed to a high *p*H for any significant period. Two other points are significant in this regard. First, when the incubation system was *p*H 5 or less, spontaneous breakdown of the substrate occurred even in the absence of the cell homogenate, indicating that at low *p*H the substrate is denatured. That a similar effect was absent above *p*H 7.5 suggests relative stability of collagen in dilute alkali. Second, results identical with those in Table 1 were obtained when the C^{14}-proline-labeled substrate collagen was purified from guinea pig skin by the method of Gross and Lapiere (*2*). It was of interest that both collagen substrates were slowly degraded by trypsin, the soluble collagen somewhat less so than the bone col-

lagen. However, in both cases the trypsin degraded the substrate less than one-third as rapidly as equivalent amounts of collagenase or cell homogenate under the conditions of these experiments.

These data indicate the existence in bone cells of an enzyme activity capable of breaking down the collagen of bone matrix at least to ultrafilterable fragments. The physiological implications of these findings are of obvious interest and raise questions concerning the site of synthesis and storage and mode of release of this activity in skeletal tissue. In preliminary experiments in which intact bone fragments were incubated in vitro for prolonged periods in nonradioactive media after labeling of their collagen with radioactive proline, the collagen activity remained unchanged for at least 48 hours (Fig. 1). Apparently, the cells in areas of bone where matrix synthesis is in progress either do not contain or do not release this enzyme activity.

In addition, more vigorous disruption of isolated cells from bone increases the relative activity of the homogenate suggesting that the enzyme may be contained in subcellular organelles, perhaps lysosomes, and is released or activated by mechanisms not yet understood. The results of preliminary experiments indicating that all the activity is in the particulate fraction of the cell homogenate support this view.

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References and Notes

1. I. Mandl, *Advan. Enzymol.* **23**, 164 (1961); D. E. Oken and R. J. Boncek, *Proc. Soc. Exptl. Biol. Med.* **96**, 367 (1951).
2. J. Gross and C. M. Lapiere, *Proc. Natl. Acad. Sci. U.S.* **48**, 1014 (1962).
3. B. Flanagan and G. Nichols, Jr., *J. Biol. Chem.* **237**, 3686 (1963).
4. ———, *Federation Proc.* **22**, 553 (1963).
5. ———, *Endocrinology*, in press.
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Transcription in vivo of DNA from Bacteriophage SP8

Abstract. *The DNA of bacteriophage SP8, when denatured, yields two components differing in buoyant density in cesium chloride gradients and separable by chromatography on a column of methylated bovine serum albumin and kieselguhr. The denser of the two strands (H) contains more pyrimidines and fewer purines than the lighter (L) strand. Only the H strand forms hybrids with the RNA synthesized by the infected host. The L strand is capable of annealing with complementary RNA synthesized in vitro with it as primer in reactions catalyzed by RNA polymerase. During the vegetative development of phage, host-specific messenger RNA is also synthesized.*

The properties of messenger RNA (mRNA) have been deduced from a variety of systems. Bautz has presented evidence that mRNA specific for bacteriophage may be single-stranded (*1*). Genetic evidence, obtained by Champe and Benzer (*2*) with the same coliphage system, suggests that the template for useful synthesis of RNA is one of the two complementary strands from bacteriophage DNA. However, experiments in vitro show that both strands of DNA are copied by the DNA-primed RNA polymerase (*3*). The availability of bacteriophages whose DNA's yield strands which can be fractionated and identified by their different buoyant densities has made it possible to test directly whether the RNA formed after phage infection is complementary to one or both of the strands of the bacteriophage DNA.

We have selected for study bacteriophage SP8 (*4*) which is virulent for *Bacillus subtilis* Marburg. This phage produces prompt and reproducible lysis of host suspensions grown in broth and synthetic media, and it yields clear plaques on solid media. By chemical analysis, the base composition of its DNA is similar to that of its host; the combined content of guanine and cytosine is 43 percent (*5*). When this DNA is heated (denatured), the separate strands show bands at distinctly different densities in the CsCl gradient. The separated strands can be fractionated by chromatography on a column containing methylated bovine serum albumin and kieselguhr by selective, discontinuous elution with saline-phosphate (*6*). The buoyant densities of this DNA in the native form and of its fractionated complementary strands, L and H, are 1.743, 1.755, and 1.764 g/ml (*5*). Both strands of DNA from phage SP8 can serve in vitro as templates for the RNA polymerase from *Escherichia coli*; from the incorporation of labeled ribonucleotides, it is apparent that the strand with the light buoyant density (the L strand) differs in base composition from the strand with heavy buoyant density (the H strand), but that they are complementary in their base composition (*7*). As in the case of bacteriophage α (*8*), the H strand is relatively rich in pyrimidines and the L strand is relatively rich in purines.

That RNA complementary to one of the strands of DNA from bacteriophage SP8 is synthesized in infected *Bacillus subtilis* was demonstrated by the principle of the DNA-RNA hybridization (*9*) which has been used for the isolation and identification of specific mRNA. In our case, the separation induced by denaturation and subsequent purification of the complementary strands permitted us to ascertain which of the strands served as a template for the synthesis of complementary RNA in vivo. The hybrid DNA-RNA molecules prepared by incubation of the DNA and RNA at 57°C (annealing) were separated by preparative CsCl density gradient centrifugation (*9*) and also by the use of an agar gel containing denatured DNA (*10*).

Labeled RNA isolated from SP8-infected *Bacillus subtilis* was first hybridized by annealing it with the H and with the L strands (SP8 DNA) previously separated chromatographically on the methylated bovine serum albumin column. After preparative centrifugation of the hybridization mixture in a CsCl density gradient, drops were collected from the bottom of the punctured centrifuge tube and the acid-precipitable fraction was assayed for radioactivity (after incubation either with or without ribonuclease). In Fig. 1 the extent of hybridization of the H and L strands of SP8 DNA with RNA from phage-infected *B. subtilis* is compared. Results obtained from samples treated with ribonuclease indicate clearly that only the RNA associated with the H strand has the resistance to ribonuclease which is expected of DNA-RNA hybrids (*11*). The amount of radioactivity in the fraction, resistant to ribonuclease and precipitable by acid, that is renatured with the H strand represents 10 to 15 percent of the labeled RNA added to the annealing mixture. On the other hand, the radioactivity associated with