Park, C. R., J. Biol. Chem., 1961, v236, 262.

10. Weil, R., unpublished data.

11. Wollenberger, A., Ristau, O., Schoffa, G., Pflügers Arch. ges. Physiol., 1960, v270, 399.

12. Bergmeyer, H. U., Editor, Methods of Enzymatic Analysis, 1963, p134.

13. Randle, P. J., Ann. Rev. Physiol., 1963, v25, 291.

14. Newsholme, E. A., Randle, P. J., Manchester, K. L., Nature, 1962, v193, 270.

15. Garland, P. B., Randle, P. J., ibid., 1963, v199, 381.

16. Garland, P. B., Randle, P. J., Newsholme, E. A., Nature, 1963, v200, 169.

17. Parmeggiani, A., Bowman, R. H., Biochem. & Biophys. Res. Comm., 1963, v12, 268.

18. Ellis, S., Pharmacol. Rev., 1956, v8, 485.

19. Ikkos, D., Luft, R., Gemzell, C. A., Almquist, S., Acta Endocrinol., 1962, v39, 547.

20. Henneman, D. H., Henneman, P. H., J. Clin. Invest., 1960, v39, 1239.

Received July 19, 1965. P.S.E.B.M., 1965, v120.

Collagenolytic Activity During Active Bone Resorption in Tissue Culture.* (30611)

E. J. KAUFMAN,† M. J. GLIMCHER, G. L. MECHANIC AND P. GOLDHABER‡ (Introduced by R. O. Greep)

Departments of Pediatric Dentistry, Oral Histopathology and Periodontology, Harvard School of Dental Medicine, and Department of Orthopedic Surgery, Harvard Medical School and Massachusetts General Hospital, Boston

Previous work has demonstrated that the resorption of bone in tissue culture(1,2) is accompanied by degradation of bone collagen (3,4). Based on the nature of the degradation products and the specificity of the enzymes present in the tissue culture medium, it was proposed that an enzyme (or enzymes) was liberated during bone resorption which possessed collagenolytic activity(4). A collagenolytic factor has been recently shown to be present in the bones of animals previously given parathyroid extract(5). In the present study, purified, reconstituted H^3-proline- and H^3-hydroxyproline-labeled collagen fibrils were added to an *in vitro* culture system. These experiments provided direct evidence that a collagenolytic factor is released during active bone resorption.

Experimental. Preparation of H^3-hydroxyproline and H^3-proline-labeled guinea pig skin collagen. Guinea pigs weighing approximately 150 g to 175 g were injected intraperitoneally with 3 millicuries of chromatographically pure H^3-proline after they had shown a consistent weight gain for 3-4 days. A second injection of 3 millicuries of H^3-proline was administered intraperitoneally 3 hours later and the animals were sacrificed 9 hours after the first injection. The skins were removed in the cold room (2°C) and kept cold during mincing. They were extracted with cold, 1 M NaCl solution, 0.05 M Tris, pH 7.4 (15 ml/g wet weight of tissue) 48 hours. The supernatant was separated from the skin residue by filtration through several layers of cheese cloth, filtered through medium- and then fine-sintered glass filters and completely clarified by centrifugation at 30,000 rpm for 3 hours in a Spinco Model L Ultracentrifuge. The clear, cold viscous supernatant was then acidified to pH 3.0 with acetic acid which resulted in a flocculent precipitate. This was centrifuged at low speed, resuspended in 0.3 M acetic acid and dialyzed at 2°C against 0.3 M acetic acid. The solution was clarified by ultracentrifugation as described and the clear supernatant made to a final concentration of 5% NaCl by the slow addition of concentrated NaCl solution. The resultant flocculent precipitate was harvested by low speed centrifugation, resuspended in 0.3 M acetic

* This investigation was supported in part by grants from USPHS, Nat. Inst. Health (DE-01298, AM-06375), and John A. Hartford Foundation, Inc.

† Supported by USPHS Training Grant, Nat. Inst. Dental Research, Nat. Inst. Health (5-T1-DE-112).

‡ Supported by USPHS Career Development Award.

acid, dialyzed against acetic acid and clarified, and the salt precipitation repeated. The protein was further purified by 3 additional reconstitutions at 2°C by dialysis against low ionic strength salt solutions at pH 7.4 (6).

Tissue culture technique. The calvaria of 5-day-old Swiss albino mice of the Webster strain were removed aseptically and the frontal and parietal bones were dissected free and placed on rectangular glass coverslips (1, 2). A small amount of H^3-hydroxyproline- and H^3-proline-labeled collagen fibrils, reconstituted by low ionic strength dialysis, was placed on the concave surface of the bone, and both the fibrils and bone were covered with a chicken plasma clot. Two ml of a nutrient medium containing heated horse serum and Gey's balanced salt solution (8:2) were added to each culture and the tubes were briefly gassed with a mixture of 50% O_2 and 50% N_2. The collagen fibrils used in each roller tube contained a total of approximately 150,000 DPM, corresponding to approximately 0.1 mg of collagen. The tubes were divided into several groups. Group I received no additional material. To Group II was added parathyroid extract (PTE) (Eli Lilly Co.) to a final concentration of 0.5 unit of parathyroid hormone per ml of medium. Group III received 10 units of heparin/ml, and to Group IV were added both PTE (0.5 unit/ml) and heparin (10 units/ml). The tissue culture media were changed every 2 days, at which time the cultures were gassed, refed and the same amount of PTE and heparin added to the appropriate tubes. The cultures were maintained for 14 days and the living cultures examined microscopically each day. The media removed from each group was either pooled for the entire 14-day experimental period or each 2-day period analyzed separately.

As control groups, collagen fibrils were incubated under identical conditions with the tissue culture medium, with tissue culture medium to which PTE (0.5 unit of parathyroid hormone/ml), heparin (10 units/ml), and both PTE and heparin were added. In addition collagen fibrils were incubated for 14 days under identical conditions with tissue culture medium containing 0.2 mg of twice crystallized trypsin (Mann Research Laboratories) and 0.005 M $MgCl_2$. As a further control collagen fibrils were incubated with trypsin in 0.1 M Tris (hydroxymethyl) aminoethane (Tris), 0.005 $MgCl_2$, pH 7.6, in order to rule out the presence of trypsin inhibitor in the tissue culture medium.

Analysis of collagen fibril degradation. The tissue culture media were brought to 2°C and trichloroacetic acid (TCA) added to a final concentration of 15% to precipitate the serum proteins and any solubilized collagen or gelatin molecules or high molecular weight peptides. The solution was clarified by centrifugation at 12,000 rpm for 1 hour in a refrigerated International Centrifuge (Model HR-1). The precipitate was washed with cold 15% TCA and the clarified washings added to the clear supernatant. Both the precipitate and the supernatant were exhaustively extracted with ether to remove the TCA. The precipitate was hydrolyzed in triple-distilled, 6N HCl at 105°C for 24 hours. The supernatant was either analyzed directly or hydrolyzed as described. The total DPM in all of the samples was counted directly in an automatic liquid scintillation counter, or the H^3-hydroxyproline and H^3-proline counted separately after hydrolysis and separation on a column of Dowex 50 (H^+ form, X–12) resin (3). Since preliminary analyses showed the same results whether the total DPM (H^3-hydroxyproline and H^3-proline) or the H^3-hydroxyproline DPM alone was used, the former was employed for the remainder of the experiments.

Characterization of the H^3-hydroxyproline and H^3-proline released into the tissue culture media during active bone resorption. Aliquots of the media were clarified either by filtering through fine-sintered glass filters, or by centrifugation after the addition of TCA to a final concentration of 15%. After the TCA was removed from the appropriate samples by extraction with ether, aliquots of the clarified media were adjusted to pH 4.4 in pyridine-acetic acid buffer, heated to 50° for 15 minutes and filtered through columns of Sephadex G-25 and G-50 resin. Other portions of the media were chromatographed

directly on columns of Dowex 50 (H^+ form, X–12) resin and the H^3-hydroxyproline and H^3-proline existing as the free amino acids separated from those in peptide-bound form (3). The percentage of free and peptide-bound H^3-hydroxyproline and H^3-proline was computed from the total H^3-hydroxyproline and H^3-proline content of the media determined after hydrolysis of the media in 6N HCl at 105°C for 24 hours and separation of the amino acids on columns of Dowex 50 (H^+ form, X–12) resin.

Results. Morphological observations of the living tissue cultures revealed active bone resorption in the groups subjected to 50% O_2 tension alone which was enhanced by addition of PTE to the tissue culture media, and even more so by addition of PTE in combination with heparin. The addition of heparin alone to the tissue culture medium did not appear to increase the amount of resorption.

After hydrolysis of several of the calvaria and residual collagen fibrils and of several aliquot parts of the tissue culture media, the total DPM present in the hydrolysate was recovered from a column of Dowex 50 (H^+ form, X–12) resin as H^3-hydroxyproline and H^3-proline.

The findings noted morphologically were confirmed by analyses of the amount of H^3-hydroxyproline and H^3-proline-TCA-soluble peptides and free amino acids(4) released from the reconstituted collagen fibrils into the tissue culture medium (Tables I and II). That the degradation of the collagen fibrils was secondary to the processes accompanying bone resorption and not the result of either the external environment (50% O_2) or the PTE or heparin added is demonstrated by the lack of collagen degradation when incubated under identical conditions in the absence of the resorbing bone. Moreover, the failure of trypsin to release significant amounts of H^3-proline and H^3-hydroxyproline when incubated under identical conditions, either in tissue culture media or in Tris buffer, demonstrates that the collagen was present in its native, undenatured state.

The rate at which the reconstituted collagen fibrils were degraded is shown in Table II. In the rapidly resorbing system utilizing PTE, heparin and 50% O_2, the peak of activity occurred at the sixth day.

TABLE I. Recovery of H^3-Hydroxyproline and H^3-Proline from Reconstituted Collagen Fibrils in the Clarified Tissue Culture Medium After 14 Days of Incubation. Exp A and B are 2 separate experiments. Values given represent the average of 2 separate analyses each of which utilized 2 cultures. All cultures incubated at 50% O_2 tension at 37°C.

	Experimental group	% of total DPM (H^3-hydroxyproline & H^3-proline) in reconstituted collagen fibrils recovered in clarified (TCA soluble) tissue culture medium
Exp A	50% O_2	11.2
	Control*	.04
	PTE + heparin	50.0
	Control*	.06
	Fibrils, medium & trypsin	.33
Exp B	50% O_2	18.2
	Control*	.5
	PTE	57.8
	Control*	.9
	Heparin	20.6
	Control*	3.4
	PTE + heparin	74.7
	Control*	1.0

* Collagen fibrils, medium and appropriate additive in absence of the calvarium.

Analysis of the media obtained during the rapid resorption induced by PTE and heparin revealed that approximately 40% of the H^3-hydroxyproline and H^3-proline DPM were in the form of the free amino acids and 60% in the form of peptides. Approximately 50% of the peptides were retarded on columns of Sephadex G-25 (molecular weight $<3,500$) and 95% retarded on columns of Sephadex G-50 (molecular weight $<8,000$ to 10,000). These results confirm those obtained by the TCA precipitation method and those reported earlier on the H^3-hydroxyproline and H^3-proline released directly from the bone collagen during active bone resorption(4). Moreover, the finding of small peptides and free amino acids indicates that the degradation of the reconstituted collagen fibrils occurred as a result of a collagenolytic factor being released from the bone during active resorption, rather than a solubilization of the undenatured protein or denatured gelatin, since

TABLE II. Recovery of H^3-Hydroxyproline and H^3-Proline from Reconstituted Collagen Fibrils in Clarified Tissue Culture Medium During 14 Days of Incubation. Values represent one experiment which utilized 2 calvaria for each experimental group. Cultures were incubated at 50% O_2 tension at 37°C.

Exp group	Day	Total DPM in collagen fibrils in culture	DPM recovered in media		% of *available* DPM from reconstituted collagen fibrils recovered in TCA supernatant
			TCA precipitate	TCA supernatant	
50% O_2	2	337,350	5,250	4,125	1.2
	4	327,975	2,500	7,725	2.4
	6	317,750	2,475	14,700	4.6
	8	300,575	2,050	17,175	5.7
	10	281,350	1,600	19,275	6.9
	12	260,475	875	14,850	5.7
	14	244,775	750	14,025	5.7
PTE + heparin	2	280,675	3,625	7,500	2.7
	4	269,550	5,975	46,500	17.2
	6	217,075	8,100	67,050	30.9
	8	141,925	3,550	35,625	25.1
	10	102,750	1,625	21,000	20.4
	12	80,125	1,125	13,425	16.7
	14	65,575	725	8,850	13.5

In the 50% O_2 group over the 14-day experimental period a total of 91,875 DPM were recovered in the TCA supernatant of the media, representing 27.2% of total DPM originally placed in the cultures. Similarly in the PTE + heparin group 199,950 DPM were recovered in the TCA supernatant, representing 71.2% of total DPM placed in culture.

the enzymes present in the tissue culture media do not degrade native collagen, gelatin, or the partially degraded gelatins produced by trypsin or pepsin, to small peptides or to the free amino acids(4).

Discussion. The results presented here demonstrate that the active resorption of bone in tissue culture, induced by a variety of agents, is accompanied by release of a collagenolytic factor which is capable of degrading undenatured collagen fibrils at near neutral pH and at 37°C. As pointed out by Walker *et al*(5), the extent of denaturation of the collagen substrate used in experiments to test for collagenolytic activity may be assessed by its susceptibility to degradation by trypsin, since the action of this enzyme results in a breakdown of native collagen molecules of less than 10%. In the experiments reported here, incubation of the reconstituted collagen fibrils with trypsin resulted in the release of less than 1% of the H^3-labeled proline and hydroxyproline. This is consistent with the known action of trypsin on native collagen, which releases a small number of peptides relatively free of the imino acids(7). It is clear, therefore, that the breakdown of the collagen fibrils was not the result of non specific proteases acting on denatured collagen, and that the criteria outlined by Walker *et al*(5) for the detection of physiological collagenolytic activity have been met.

Earlier studies employing the same tissue culture technique had provided indirect evidence for a collagenolytic factor in bone by demonstrating the release of both free and peptide-bound hydroxyproline from the bone collagen into the tissue culture medium during active bone resorption(3,4). In comparing the results of these previous experiments with the present ones, it is clear that the extent of bone resorption as measured by the release of hydroxyproline from the bone collagen is paralleled by the amount of collagenolytic activity released. This was true not only for the overall or total 14-day experimental test period, but also with respect to the time sequence as measured at 2-day intervals. The results also confirm the previous findings(2,3,4,8) that parathyroid extract enhances bone resorption by a direct action on bone tissue, and suggests that at least part of the mechanism of action of parathyroid hormone is to increase the production and release of an enzyme (or enzymes) which possesses collagenolytic activity. This action is enhanced by addition of heparin, but as noted

morphologically(9), heparin alone does not appear to have any effect. However, since heparin has been shown in tissue culture to markedly increase the amount of bone resorption with suboptimal concentrations of parathyroid extract(9) and since the results presented here also demonstrate an enhancement of resorption by heparin in the presence of parathyroid extract, the possibility remains that patients on long term heparin therapy who have a normal amount of circulating parathyroid hormone might develop either osteoporosis or show a marked increase in bone turnover rate. Indeed, the occurrence of osteoporosis in such patients has recently been reported(10).

It is not possible to tell whether the increased resorption noted with parathyroid extract results from an increased synthesis of the enzyme per cell, or whether the hormone induces the differentiation of cells capable of producing the enzyme. Microscopic observation of the living tissue cultures under similar conditions(2) has clearly shown the sudden and dramatic appearance of osteoclasts in association with the resorbing bone margin. It remains to be seen, however, whether these cells are in fact the ones which synthesize the collagenolytic enzyme.

A collagenolytic factor has been previously demonstrated in the bones of animals which had been treated with parathyroid hormone prior to sacrifice by incubating the bones with undenatured collagen gels *in vitro*(5). These studies differ somewhat from the present ones in demonstrating that bone which had been undergoing resorption *in vivo* as a result of parathyroid extract injection, degraded native collagen fibrils *in vitro* to a markedly greater extent than bones from control animals which had not received the hormone. The present studies demonstrate that collagenolytic activity is released from the living bone *during active bone resorption.* The results of both groups of experiments are certainly complementary, however, and the demonstration of an active collagenolytic factor in bone provides a mechanism whereby undenatured bone collagen may be removed during the remodelling process. In this respect, the same basic mechanism appears to be operative in the resorption of bone collagen as in the resorption of tadpole soft tissues during metamorphosis in which instance a collagenolytic factor has also been previously demonstrated (11).

Since bone is a tissue which is constantly undergoing remodelling, one would expect some collagenolytic activity to be present in normal bone. A small amount of collagenolytic activity was in fact demonstrated in the control animals by Walker *et al*(5), utilizing the technique of culturing the bone directly on collagen gels *in vitro,* but they could not confirm the earlier report of Wood and Nichols(12) that such activity was present in homogenates obtained from bone.

Summary. A tissue culture system has been used to study the release of collagenolytic activity from actively resorbing bone by measuring the degradation of purified, reconstituted H^3-hydroxyproline- and H^3-proline-labeled collagen fibrils. Under conditions where active bone resorption was observed morphologically, a collagenolytic factor was liberated from the bone which degraded reconstituted, undenatured collagen fibrils. This collagenolytic activity was increased by addition of parathyroid extract to the tissue culture medium, and still further enhanced when both parathyroid extract and heparin were added.

The authors gratefully acknowledge the technical assistance of Mrs. Josefina Gonzalez and Miss Joan Jennings.

1. Goldhaber, P., Excerpta Med., 1962, v16, 811.

2. ———, in Mechanisms of Hard Tissue Destruction, R. F. Sognnaes, ed., Pub. 75, A.A.A.S., Washington, D.C., 1963, 609.

3. Stern, B. D., Mechanic, G. L., Glimcher, M. J., Goldhaber, P., Biochem. Biophys. Res. Comm., 1963, v13, 137.

4. Stern, B. D., Glimcher, M. J., Mechanic, G. L., Goldhaber, P., Proc. Soc. Exp. Biol. and Med., 1965, v119, 577.

5. Walker, D. G., Lapiere, C. M., Gross, J., Biochem. Biophys. Res. Comm., 1964, v15, 397.

6. Glimcher, M. J., Francois, C. J., Richards, L., Krane, S. M., Biochim. Biophys. Acta, 1964, v93, 585.

7. Hodge, A. J., Highberger, J. H., Deffner, O. J., Schmitt, F. O., Proc. Nat. Acad. Sci., 1960, v46, 197.

8. Gallard, P., Exp. Cell Res., 1955, Supp. 3, 154.

9. Goldhaber, P., Science, 1965, v147, 407.
10. Griffith, G. C., Nichols, G., Asher, J. D., Flanagan, B., J.A.M.A., 1965, v193, 91.
11. Lapiere, C. M., Gross, J., in Mechanisms of Hard Tissue Destruction, R. F. Sogonnaes, ed., Pub. 75, A.A.A.S., Washington, D. C., 1963, 663.
12. Wood, J. F., Nichols, G., Science, 1963, v142, 386.

Received July 22, 1965. P.S.E.B.M., 1965, v120.

Effect of Propylthiouracil on Preference Threshold of Rats for NaCl Solutions.*† (30612)

MELVIN J. FREGLY AND IRVING W. WATERS
Department of Physiology, University of Florida, College of Medicine, Gainesville

Hypothyroidism, produced either by thyroidectomy or treatment with antithyroid drugs, induces a spontaneous salt appetite in rats given choice between water and .15 M NaCl solution to drink(1,2). The salt appetite is reminiscent of that commonly observed in adrenalectomized rats by a number of investigators(3,4). Hypothyroid rats also resemble adrenalectomized rats in that they are unable to conserve sodium when the diet is deficient in this element and die (50%) within 3 weeks(5). In addition, intraperitoneal loading with either saline or water results in greater urinary loss of sodium and chloride after 5 hours than is observed for normal controls(6). Since the resemblance of hypothyroid to adrenalectomized rats is so striking, it was deemed worthwhile to test the preference threshold of hypothyroid rats for NaCl solutions to determine if it is reduced as is reported for adrenalectomized rats(4,7).

Methods and results. Two separate experiments were performed. Each utilized male rats of the Carworth CFN strain. The conditions of both experiments were similar in that the rats were kept in individual cages in a windowless room in which temperature was maintained at 26 ± 1°C and illumination was from 8 A.M. to 6 P.M. Each rat was provided with finely ground Rockland Rat Diet in spillproof containers described in detail elsewhere(8). Two containers consisting of cast aluminum fountains and nursing bottles provided drinking fluid(9).

Experiment 1. Six control and 6 propylthiouracil-treated (PTU)[2] rats were used. The PTU-treated rats received the drug in food (1.0 g PTU/kg food) for 8 weeks prior to the experiment and were continued on the drug during the experiment. During the first 2 weeks of the experiment, both bottles contained distilled water. Intakes were measured daily to ascertain that each rat drank roughly equal amounts of water from each bottle. Positions of the bottles on the cage were interchanged daily to avoid habit formation in selection of drinking fluid. During this period, about 20% of the rats were rejected as position drinkers and were replaced by other rats. At the end of the 2-week period, 2-day test periods were begun. During the first period each rat was offered choice between distilled water (bottle A) and .0001 M NaCl solution (bottle B). All NaCl solutions used were made from a concentrated stock NaCl solution by serial dilution. Each dilution was checked for accuracy by determination of chloride concentration (Cotlove Chloridometer). During subsequent 2-day periods, each rat was given choice between distilled water and the following molar NaCl solutions in chronological sequence: .0003, .0004, .0008, .001, .010, .020, .050, .100, .150 and .300. Daily intakes of water and NaCl solution were measured and expressed as ml/100 g BW/day to correct for differences in body weight.

The criterion of preference threshold used was similar to that of Richter(7); namely,

* Supported by Grant HE-03503-08 from Nat. Inst. Health, Bethesda, Md.

† 6-n- propyl-2-thiouracil, Nutritional Biochemicals Corp., Cleveland, Ohio.