Studies of Collagen Degradation During Bone Resorption in Tissue Culture.* (30243)

B. D. STERN, M. J. GLIMCHER, G. L. MECHANIC AND P. GOLDHABER†
(Introduced by R. O. Greep)
Department of Oral Histopathology and Periodontology, Harvard School of Dental Medicine, and Department of Orthopedic Surgery, Harvard Medical School and Massachusetts General Hospital, Boston

The resorption of bone in tissue culture has been shown to be accompanied by degradation of the bone collagen to peptides rather than its solubilization as either undenatured collagen macromolecules or denatured gelatin (1). The amount of collagen degraded and released into the tissue culture media was found to be enhanced by parathyroid hormone, demonstrating a direct action of this hormone on bone tissue resulting in the destruction of the organic matrix(1). These results suggested that the degradation of collagen during bone resorption was enzyme mediated, and that one of the actions of parathyroid hormone was to increase the production in bone cells of the enzyme (or enzymes) responsible. Furthermore, since the primary chains of undenatured collagen are resistant to hydrolysis by enzymes other than bacterial collagenase, the results implied the synthesis and production of a mammalian collagenase (or collagenases) during the resorption of bone. This hypothesis is consistent with the findings of Lapiere and Gross(2) who have demonstrated collagenolytic activity in the soft tissues of tadpoles during the active resorption of collagen which occurs in metamorphosis, and of Walker, Lapiere and Gross(3) who have demonstrated the presence of collagenolytic activity in the long bones of rats after administration of parathyroid hormone *in vivo*. While little or no activity was found in normal long bones by these workers, Woods and Nichols(4) have recently reported the presence of collagenolytic activity in bone-cell homogenates.

In the present study the degradation of bone collagen during active resorption has been studied in tissue culture with particular emphasis on the state of the hydroxyproline released into the tissue culture medium. The results are consistent with the conclusion that collagen is degraded by an enzyme (or enzymes) whose action is similar to that of bacterial collagenase.

Experimental. Tissue culture technique. Five-day-old Swiss albino mice were injected intraperitoneally with 0.5 mc of H^3-proline and sacrificed 18 hours later. The frontal and parietal regions of the calvaria were dissected free and set up as roller-tube cultures(5,6). The tubes were all briefly gassed with a mixture of 50% oxygen and 50% nitrogen, and parathyroid extract‡ (PTE) added to half of the tubes at a final concentration of 0.5 unit of parathyroid hormone per ml of medium. The tissue culture media were changed every 2 days, at which time the cultures were refed, the same amount of parathyroid extract added to the appropriate tubes, and the cultures gassed. The cultures were maintained for 14 days and examined microscopically each day. The medium removed from the 4 or 5 tubes of each group was pooled for each 2-day period. At the end of 14 days the calvaria were removed from the roller tubes and pooled for analysis.

Isolation of hydroxyproline. The tissue culture media were brought to known volume and aliquots cooled to 2°C. Protein and high molecular weight peptide-bound, low molecular weight peptide-bound, and free hydroxyproline were separated by several different procedures (Fig. 1). Cold perchloric acid, 0.9 M, was added to a final concentration of 0.3 M, and the precipitate separated by centrifugation at 12,000 to 15,000 rpm for 10 to 15 minutes. The precipitate was then washed

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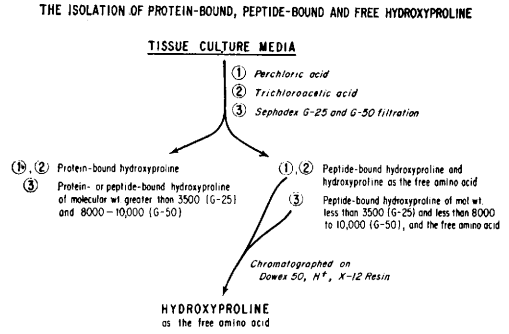

FIG. 1. Schema showing methods used to separate protein-bound, peptide-bound and free hydroxyproline.

with cold 0.3 M perchloric acid, separated by centrifugation and the washings added to the original supernatant. The perchloric acid was removed by neutralization with potassium hydroxide, and after filtering the precipitate the procedure was repeated on the supernatant several times. The final clear supernatant was dried.

The proteins were also precipitated with trichloroacetic acid at a final concentration of 15%, washed with cold trichloroacetic acid, and the trichloroacetic acid removed with ethyl ether. Other aliquots of the media were heated to 50°C for 10 minutes and chromatographed on 1.8 cm × 40 cm columns of Sephadex G-25 and G-50 resin equilibrated with pyridine-acetate buffer. The eluates from the Sephadex resin columns, corresponding to material with molecular weights greater than approximately 3,000 to 3,500 (G-25), and 8,000 to 10,000 (G-50), were separated from those of lower molecular weight. Both the Sephadex G-25 and G-50 columns were calibrated by chromatographing hemoglobin under identical conditions.

The total hydroxyproline content of the media of the protein and peptide fractions, and of the residual calvaria were determined colorimetrically(7) on fractions of acid hydrolysates (6 N HCl, 105° 24 hours) separated on Dowex 50-H+, X-12 resin(1). Free hydroxyproline in the media was determined colorimetrically on fractions isolated by chromatographing aliquots of the unhydrolyzed protein-free supernatants on columns of Dowex 50-H+, X-12 resin as described. The radioactivity of all of the samples was measured using an automatic liquid scintillation counter.

Enzymatic activity of the tissue culture medium-substrates:

(a) *Acid soluble calf skin collagen.* Acetic acid soluble collagen was purified by reconstitution (5 times) into fibrils followed by the trichloroacetic acid-ethanol procedure of Gross(8).

(b) *Calf skin gelatin.* Gelatin was prepared by heating solutions of purified calf skin collagen at 60°C for 15 minutes.

(c) *Pepsin digest of calf skin gelatin.* Approximately 200 mg of lyophilized calf skin gelatin was dissolved in 50 ml of 0.5 M HCl, 0.05 M sodium citrate at pH 2.0, and warmed to 50°C for 15 minutes. After the solution was cooled to 37°C, 10 mg of crystalline pepsin (Mann Research Laboratories) was added, and the mixture was incubated for 24 hours. Aliquots were removed at zero time and at the end of the experiment for ninhydrin determinations(9). At the end of the experiment the solution was frozen quickly at —70°C by placing the reaction vessel in ethylene glycol monomethyl ether (methyl cellosolve) containing dry ice. The solution was thawed at 2°C and the pepsin precipitated by the addition of cold trichloroacetic acid to a final concentration of 5%. The precipitate was removed by centrifugation and by filtration through a medium sintered glass filter. The trichloroacetic acid was removed from the supernatant with ethyl ether and the solution desalted on a column of Sephadex G-25 in pyridine-acetate and lyophilized.

(d) *Trypsin digest of calf skin gelatin.* Approximately 100 mg of lyophilized calf skin gelatin was dissolved in 25 ml of 0.1 M Tris-(hydroxymethyl)aminomethane (Tris), 0.005 M $MgCl_2$, pH 7.6 and heated for 15 minutes at 50°C. After the solution was cooled to 37°C, 5 mg of twice crystallized trypsin (Mann Research Laboratories) was added to the solution, and the mixture incubated for 24 hours at 37°C. Aliquots were removed and treated as described in section (c) above.

(e) *Trypsin digest of pepsin-digest of calf skin gelatin.* Approximately 100 mg of lyophilized pepsin-digested calf skin gelatin was dissolved in 25 ml of 0.1 M Tris, 0.005

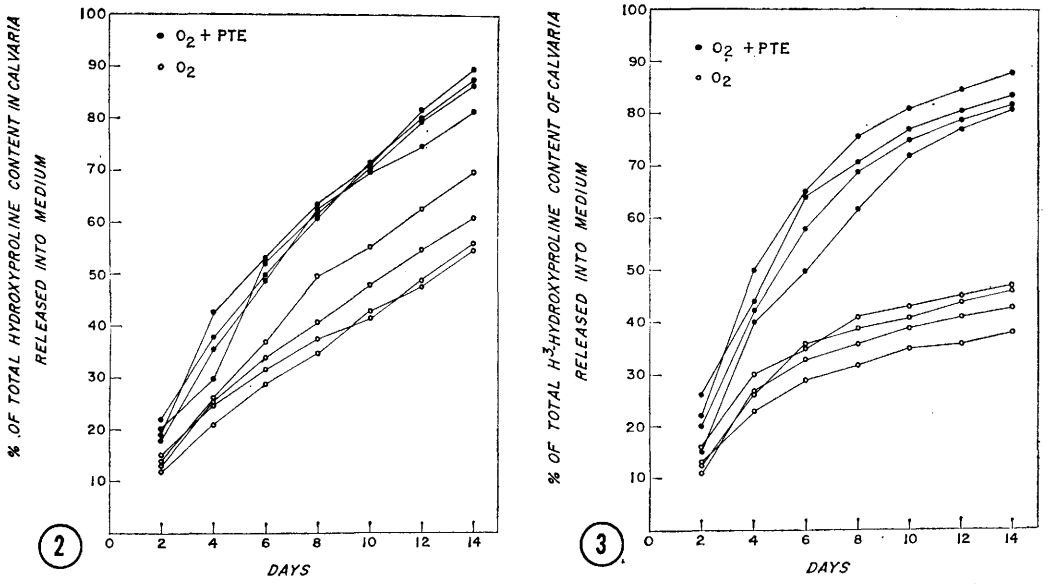

FIG. 2. Release of hydroxyproline from mouse calvaria into tissue culture media during active bone resorption. Values represent total hydroxyproline recovered after acid hydrolysis. O_2 = 50% oxygen; O_2 + PTE = 50% oxygen plus 0.5 unit of parathyroid hormone/ml of tissue culture medium.

FIG. 3. Release of H^3-hydroxyproline from mouse calvaria into tissue culture media during active bone resorption. Values represent total H^3-hydroxyproline recovered after acid hydrolysis. O_2 = 50% oxygen; O_2 + PTE = 50% oxygen plus 0.5 unit of parathyroid hormone/ml of tissue culture medium.

M $MgCl_2$, pH 7.6 and treated as described in (d) above.

(f) *Papain digest of calf skin gelatin.* Approximately 100 mg of lyophilized calf skin gelatin was dissolved in 25 ml of 0.1 M Tris, 0.02 M thiodiglycol, pH 7.4 and heated for 15 minutes at 50°C. After the solution was cooled to 37°C, 5.0 mg papain powder (Mann Research Laboratories) was added to the solution, and the mixture incubated at 37°C for 24 hours. Aliquots were removed and treated as described in (c) above.

(g) *Collagenase digests of calf skin collagen and gelatin.* Approximately 100 mg of lyophilized calf skin collagen was dissolved in 25 ml 0.1 M Tris, 0.005 M CaC_2, pH 7.4 and heated for 15 minutes at 50°C. After the solution was cooled to 37°C, 5.0 mg of bacterial collagenase (Mann Research Laboratories) further purified by the method of Seifter, Gallop, Klein and Meilman(10), was added to the solution. The mixture was incubated at 37°C for 24 hours. The pH was monitored at intervals and maintained at pH 7.4 by addition of dilute NaOH. Aliquots were removed and treated as described in (c) above, except that the solution was desalted on a 0.9 cm by 50 cm column of Dowex 50-H+, X-8 resin. Collagenase digests of collagen were obtained by incubating collagen fibrils, and collagenase under similar conditions as described.

(h) *The dipeptides, glycyl-L-hydroxyproline and L-hydroxyprolylglycine.* The dipeptides were obtained from Mann Research Laboratories.

Procedure. Each of the substrates was incubated for 48 hours at 37°C in roller tubes under the identical conditions used for the tissue culture experiments except that the calvaria were omitted. The entire tissue culture media as well as its components (horse serum, chick plasma clot and Gey's balanced salt solution) were tested separately with and without addition of parathyroid extract and with and without addition of soybean trypsin inhibitor. After 48 hours of incubation, protein-bound and free-hydroxyproline were determined as described.

Results. Active bone resorption was ob-

TABLE I. Relative Rates of Collagen Resorption from Mouse Calvaria in Tissue Culture. Each experiment (1 through 4) represents data obtained from 4 calvaria.

	Hydroxyproline content of calvaria, μg/sample				Hydroxyproline released into tissue culture media, μg/sample				% of hydroxyproline available in sample released into the media			
					Exp No.							
Days	1	2	3	4	1	2	3	4	1	2	3	4
A. 50% Oxygen alone												
0	266.4	162.1	147.8	132.2								
2	266.4	162.1	147.8	132.2	35.2	22.6	22.6	15.8	13.2	13.9	15.3	12.0
4	231.2	139.5	125.2	116.4	35.2	18.0	14.3	12.0	15.2	12.9	11.4	10.3
6	196.0	121.5	110.9	104.4	27.6	15.0	10.5	11.3	13.7	12.3	9.5	10.8
8	168.8	106.5	100.4	93.1	36.8	12.0	9.0	8.3	21.9	11.3	9.0	8.9
10	132.0	94.5	91.4	84.8	12.8	10.8	6.8	10.5	9.7	11.3	7.4	12.4
12	119.2	83.7	84.6	74.3	20.8	10.8	9.8	6.0	17.5	12.9	11.6	8.1
14	98.4	72.9	74.8	68.3	17.6	10.8	9.8	9.8	17.7	14.8	13.1	14.3
Unresorbed calvaria	80.8	62.1	65.0	58.5								
B. PTE and 50% oxygen												
0	264.4	164.3	186.4	167.2								
2	264.4	164.3	186.4	167.2	54.4	29.6	35.3	37.6	20.4	18.0	18.9	22.5
4	210.0	134.7	151.6	129.6	26.4	30.4	45.1	27.0	12.6	22.5	29.8	20.1
6	183.6	104.3	106.0	102.6	57.6	20.8	19.5	20.3	31.4	20.0	18.4	19.8
8	126.0	83.5	86.5	82.3	20.8	20.8	18.0	18.8	16.5	24.9	20.8	22.8
10	105.2	62.7	68.5	63.5	28.0	12.8	16.5	12.0	26.6	20.4	24.0	18.9
12	77.2	49.9	52.0	51.5	26.4	15.2	14.3	11.3	34.3	30.4	27.5	21.9
14	50.8	34.7	37.7	40.2	22.4	13.6	14.3	10.5	44.1	39.2	37.9	26.1
Unresorbed calvaria	28.4	21.1	23.4	29.7								

served microscopically in both the oxygen and the oxygen plus parathyroid treated cultures, but the process was markedly enhanced by addition of PTE to the tissue culture medium as demonstrated previously(6).

The morphological results were confirmed by the finding of hydroxyproline and H^3-hydroxyproline in the tissue culture media and a considerable increase in the amount of both in the cultures to which PTE had been added (Fig. 2 and 3). Approximately 87% of the total collagen and 86% of the H^3-hydroxyproline-labeled collagen present in the calvaria were resorbed respectively after 14 days in those cultures to which PTE had been added, compared to 63% and 46% resorbed in the cultures subjected only to 50% oxygen.

Since the tissue culture media were changed every 2 days and equal amounts of fresh PTE added at those times, it was also possible to calculate the percentage of the residual (and therefore available) collagen which was released during each 2-day time period. Tables I and II demonstrate that the amount of collagen which was degraded during each of the 2-day time periods was consistently higher in those cultures to which PTE had been added. The average rate of collagen resorption (micrograms hydroxyproline released/total micrograms hydroxyproline in the sample) for each 2-day period was 24.9% in the PTE treated cultures compared to 12.9% in those cultures to which no PTE was added. Similarly the rate of H^3-hydroxyproline released was 24.5% in the PTE treated cultures and 8.4% in those cultures to which no PTE was added.

A representative experiment concerned with the state of the hydroxyproline and H^3-hydroxyproline found in the media during resorption for both *oxygen* and *oxygen* plus PTE treated cultures is shown in Tables III and IV. None of the hydroxyproline in the media was protein-bound or present in peptides of molecular weight greater than approximately 3,000, since all of the hydroxyproline and H^3-hydroxyproline was recovered in fractions which were retarded on columns of Sephadex G-25. Approximately 51% of the hydroxyproline and 53% of the H^3-hydroxyproline in the PTE plus oxygen treated

cultures, and 64% of both the hydroxyproline and H^3-hydroxyproline in the oxygen treated samples was recovered as the free amino acid (Tables III and IV).

The results of incubating the tissue culture medium and its components with: (1) purified, reconstituted collagen; (2) gelatin derived from the purified collagen; (3) various peptide mixtures obtained by enzymatic digestion of the gelatins with trypsin, pepsin and papain; (4) collagenase digests of gelatin; and (5) synthetic dipeptides are shown in Table V.

Significant amounts of free hydroxyproline were released only from collagenase digests of collagen and gelatin and from the synthetic dipeptide glycyl-L-hydroxyproline after incubation with the whole tissue culture medium or with the horse serum component of the tissue culture medium. Similar results were obtained with horse serum in which the cells were separated from the plasma immediately after whole blood had been drawn from the animals.

Discussion. The release of hydroxyproline and H^3-hydroxyproline from mouse calvaria

TABLE II. Relative Rates of H^3-Collagen Resorption from Mouse Calvaria in Tissue Culture. Each experiment (1 and 2) represents data obtained from 4 calvaria.

Days	H^3-hydroxyproline content of calvaria disintegrations/min/sample		H^3-hydroxyproline released into tissue culture media disintegrations/min/sample		% of H^3-hydroxyproline available in sample released into the media	
	Exp No.					
	1	2	1	2	1	2
A. 50% oxygen alone						
0	38,357	31,922				
2	38,357	31,922	4,893	3,683	12.7	11.5
4	33,484	28,239	5,210	4,518	15.5	16.0
6	28,274	23,721	3,400	2,490	12.0	10.5
8	24,874	21,231	2,870	1,004	11.5	4.7
10	22,004	20,227	845	886	3.8	4.4
12	21,159	19,341	894	670	4.2	3.5
14	20,265	18,671	686	529	3.4	2.8
Unresorbed calvaria	19,579	18,142				
B. PTE and 50% oxygen						
0	24,227	37,878				
2	24,227	37,878	6,419	8,347	26.5	22.1
4	17,808	29,531	4,224	10,670	23.7	36.1
6	13,584	18,861	5,086	5,513	37.4	29.2
8	8,478	13,348	1,580	4,347	18.6	32.5
10	6,898	9,001	1,428	1,997	20.7	22.1
12	5,470	7,004	976	1,505	17.8	21.4
14	4,494	5,499	627	857	13.9	15.6
Unresorbed calvaria	3,867	4,642				

TABLE III. State of Hydroxyproline Released into the Tissue Culture Media During Active Bone Resorption. Values expressed as μg hydroxyproline per sample.

	Protein-bound*		Peptide-bound		Free amino acid		% as free amino acid	
Day	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE
2	0	0	12.8	18.4	9.8	11.2	43	38
4	0	0	3.0	16.0	15.0	14.4	83	47
6	0	0	4.0	6.4	11.0	14.4	73	69
8	0	0	4.8	11.2	7.2	9.6	60	46
10	0	0	3.6	5.6	7.2	7.2	67	56
12	0	0	3.6	6.4	7.2	8.8	67	58
14	0	0	4.2	5.6	6.6	8.0	61	59

* Samples separated on Sephadex G-25 contained no hydroxyproline in the fraction corresponding to material of molecular weight greater than 3,000 to 3,500.

into the tissue culture medium provides direct chemical evidence that collagen is removed from bone during active resorption. Furthermore, it is clear from the findings that parathyroid hormone has a direct action on bone tissue, and that one of the results of

TABLE IV. State of H^3-Hydroxyproline Released into Tissue Culture Media During Active Bone Resorption. Values are expressed as disintegrations per minute per sample.

	Protein-bound*		Peptide-bound		Free amino acid		% as free amino acid	
Day	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE	50% O_2	50% O_2 + PTE
2	0	0	1,548	3,753	2,135	4,594	58	55
4	0	0	1,446	6,191	3,072	4,479	68	42
6	0	0	1,146	2,208	1,344	3,305	54	60
8	0	0	253	2,001	751	2,346	75	54
10	0	0	245	655	641	1,342	72	67
12	0	0	125	512	545	993	81	66
14	0	0	156	240	373	617	70	72

* Samples separated on Sephadex G-25 contained no radioactivity in the fraction corresponding to material of molecular weight greater than 3,000 to 3,500.

TABLE V. Release of Hydroxyproline as Free Amino Acid from Various Substrates by Components of Tissue Culture Medium.

Substrates	μg of hydroxyproline in sample	Incubation medium	μg of hydroxyproline released as free amino acid	% of hydroxyproline released as free amino acid during incubation
Reconstituted calf skin collagen	1,010	WTM*	4	<1
Calfskin gelatin	1,060	WTM	6	<1
Pepsin digest of calfskin gelatin	1,015	WTM	12	1
Trypsin digest of calfskin gelatin	990	WTM	15	<2
Pepsin-trypsin digest of calfskin gelatin	1,012	WTM	21	2
Papain digest of calfskin gelatin	1,003	WTM	18	<2
Collagenase digest of calfskin gelatin	1,050	WTM	440	42
	1,023	HS†	468	46
	52	HS	36	69
	1,060	Clot‡	101	10
	50	Clot	6	12
	48	Gey's solution	1	2
Glycyl-L-hydroxyproline	978	HS	525	54
	48	HS	29	60
	948	Clot	9	<1
	53	Clot	2	4
	961	Gey's solution	4	<1
L-hydroxyprolylglycine	1,070	HS	120	11
	57	HS	6	11
	53	Clot	2	4
	1,050	Gey's solution	10	<1

Neither addition of 0.5 U. PTE/ml culture medium nor addition of trypsin inhibitor had any effect on the results of substrate incubation.

Analysis of a collagenase digest of calfskin gelatin prior to incubation with Whole Tissue Culture Medium indicated the presence of less than approximately 2% free hydroxyproline.

* WTM = Whole tissue culture medium.

† HS = Horse serum.

‡ Clot = Chicken plasma clot.

this action includes an increase in the rate at which bone collagen is removed.

The identification of hydroxyproline in the form of the free amino acid and in peptides of relatively low molecular weight, combined with the findings that incubation of collagen or gelatin with the whole tissue culture medium and its components does not release free hydroxyproline, suggests that the bone collagen is enzymatically degraded during resorption and is not merely solubilized as collagen or gelatin and then secondarily digested by enzymes present in the tissue culture medium.

Some insight as to the type of collagenolytic degradation which occurs during bone resorption and the nature of the peptides released into the tissue culture medium may be gained from the experiments in which various enzymatic digests of collagen and gelatin were incubated with the tissue culture medium. Table V indicates that there are enzymes present in the tissue culture medium (primarily in the horse serum) that can degrade the peptides produced by the action of collagenase on collagen or gelatin and the dipeptide glycyl-L-hydroxyproline to the extent of releasing approximately 50% to 70% of the hydroxyproline as the free amino acid, but which do not degrade the peptides produced by the action of pepsin, trypsin or papain on gelatin. This suggests that the enzymatic degradation of bone collagen during active resorption produces peptides whose size and amino acid sequence are similar to those produced by bacterial collagenase. Such peptides would then be further degraded by the enzymes present in the tissue culture medium, so that a considerable proportion of the hydroxyproline would be released as the free amino acid.

The proposal that bone cells produce a collagenase during bone resorption is consistent with the findings of Walker, Lapiere and Gross(3) that the bones of animals which had received parathyroid extract prior to sacrifice had collagenolytic activity when incubated with purified, reconstituted collagen fibrils *in vitro*.

Summary. A tissue culture system has been used to study the removal of bone collagen from radioactively labeled, resorbing mouse calvaria, by measuring the amount of hydroxyproline and the radioactivity of H^3-hydroxyproline released into the tissue culture medium. The amounts of hydroxyproline and H^3-hydroxyproline released from the calvaria were enhanced by addition of parathyroid extract to the medium and correlated with the extent of bone resorption observed microscopically in the living cultures. Evidence is presented which indicates that bone collagen is partially degraded during active bone resorption in tissue culture to peptides similar in size and sequence to those produced by the action of bacterial collagenase on collagen.

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