THE RESORPTION OF BONE COLLAGEN IN TISSUE CULTURE*

Burt D. Stern, Gerald L. Mechanic, Melvin J. Glimcher and Paul Goldhaber†

Department of Oral Histopatholgy and Periodontolgy, The Harvard School of Dental Medicine, and The Department of Orthopedic Surgery, The Harvard Medical School, and The Massachusetts General Hospital, Boston 14, Massachusetts

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Although it is clear that the resorption of bone includes the loss of the organic matrix (primarily collagen) as well as the inorganic crystals, it is not known whether the bone collagen is solubilized, solubilized and denatured, or enzymatically digested during bone resorption. In the present investigation, 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 radioactivities of H^3-hydroxyproline and H^3-proline released into the tissue culture medium. The amounts of hydroxyproline and proline released from the calvaria were enhanced by the addition of parathyroid extract to the medium and correlated closely with the extent of bone resorption observed microscopically in the living cultures. Evidence is presented which indicates that the collagen is partially degraded into peptides during active bone resorption in tissue culture, and is not simply solubilized, or solubilized and denatured.

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EXPERIMENTAL PROCEDURE

Tissue Culture Technique.--The frontal and parietal regions of the calvaria of 6 day old Swiss albino mice, labeled in utero with H^3-hydroxyproline and H^3-proline by the intraperitoneal injection of 4 daily doses of 0.5 mc of H^3-proline to the mother just prior to parturition, were placed in roller-tube cultures (Goldhaber, 1961, 1962, In Press). The tubes were all briefly gassed with a mixture of 50% O_2 and 50% N_2, and parathyroid extract (Eli Lilly Co. added to six of the ten tubes at a final concentration of 0.5 units of parathyroid hormone per ml of medium. The tissue culture media were changed every 2 days, at which time the cultures were gassed, refed and the same amount of parathyroid extract 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 pooled for each 2 day period. The media contained no hydroxyproline.

Isolation of Hydroxyproline and Proline.--Aliquots of the pooled media were hydrolyzed in 6N HCl, 105°, 24 hours, and the hydroxyproline and proline separated by chromatography on a 0.9 cm x 10.0 cm column of Dowex 50-H^+, X-12 resin (13 to 23 micron spherical particles), equilibrated and eluted with 2N HCl at 8° to 10°. The column was run at the rate of 1.0 ml/minute. Hydroxyprolin was quantitatively recovered in the fraction eluted between 8 ml and 16 ml, and proline in the fraction eluted between 20 ml and 28 ml. The dipeptides, Gly-Hypro and Hypro-Gly, as well as collagenase digests of calf skin collagen, were chromatographed under identical conditions. Less than 1% of the hydroxyproline applied to the column was eluted in the fraction representing hydroxyproline. The small amount of hydroxyproline recovered represented free hydroxyproline originally present in the samples, since the amount of hydroxyproli in this fraction determined colorimetrically did not change after acid hydrolysis (6N HCl, 105°, 24 hours), and similar amounts could be detected colorimetrically in the original samples prior to chromatography. The fraction eluted from the column in the hydroxyproline peak was further identified by high voltage electrophoresis on 58 cm strips of Whatman No. 3 MM paper (pH 1.9, formic-acetic

acid, 5000 volts, 3.5 hours). A small strip of the paper corresponding to the region represented by authentic hydroxyproline was stained with ninhydrin, and the remainder eluted and its radioactivity determined. Considering the amount of paper stained with ninhydrin and not eluted, 90% to 95% of the counts applied to the paper were recovered in the region corresponding to hydroxyproline.

Hydroxyproline was determined colorimetrically (Stegemann, 1958) on the chromatographic fractions using smaller reagent and sample volumes, which permitted the detection of as little as 1 μgm. The radioactivities of H^3-hydroxyproline and H^3-proline were measured in an automatic liquid scintillation counter.

RESULTS

Hydroxyproline and H^3-hydroxyproline were found in the tissue culture media of both groups (Figs. 1,2). The maximum amount and specific activity of the hydroxyproline released into the media occurred during the early stages when the resorption was most intense as determined by the total amount of hydroxyproline released, as well as microscopic observation. The addition of parathyroid extract significantly increased the amount and specific activity of hydroxyproline released (Fig 3).

The release of H^3-proline into the media of both groups followed a pattern similar to that of H^3-hydroxyproline. Proline was not determined chemically because of the relatively large amounts which were present in the tissue culture media prior to incubation with the calvaria.

DISCUSSION

The findings of hydroxyproline, H^3-hydroxyproline and H^3-proline in the tissue culture media provides direct chemical evidence that collagen was being removed from the calvaria while active bone resorption was observed microscopically. The process was enhanced by the addition of parathyroid extract, demonstrating that the hormone has a direct action on bone tissue (Gaillard, 1955), the result of which includes the resorption of bone collagen.

This action was presumably mediated via the bone tissue cells, since parathyroid extract did not have a similar effect on collagen fibrils or gelatin solutions incubated in tissue culture medium.

In view of the fact that the calvaria were labeled *in utero* and the animals sacrificed at six days of age, the collagen containing hydroxyproline of high specific activity probably represented the older collagen synthesized while the animal was *in utero*. The high specific activity of the hydroxyproline released during the early phases of resorption, especially in the parathyroid extract-treated samples, indicates that the older collagen was removed first. This is consistent with the finding that the parathyroid hormone acts on the older, more stable bone mineral *in vivo* (Woods and Armstrong, 1956). The preferential removal of older collagen during tissue resorption may be a general

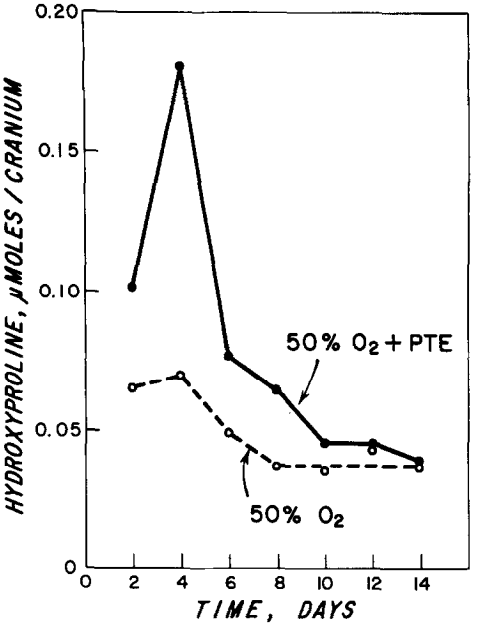

Fig. 1.--The amount of hydroxyproline released into the tissue culture media during the active resorption of bone induced by 50% oxygen tension with and without the addition of parathyroid extract (PTE). Represents the total hydroxyproline in the media after acid hydrolysis.

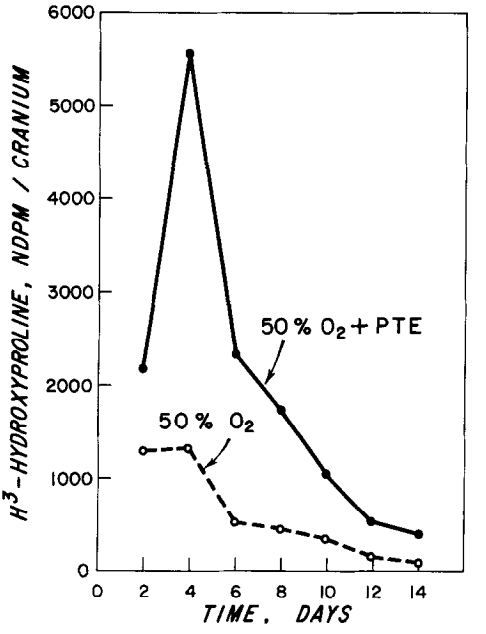

Fig. 2.--The total radioactivity of H^3-hydroxyproline released into the tissue culture media during the active resorption of bone. Represents the total H^3-hydroxyproline in the medium after acid hydrolysis.

phenomenon, in view of similar results obtained by Lapiere and Gross (In Press) studyingthe resorption of tadpole soft tissue collagen during metamorphosis.

Preliminary experiments in which the culture media was fractionated into proteins and peptides of molecular weight less than 8000 to 10,000 on Sephadex G-50 resin columns, as well as by trichloroacetic acid precipitation (final concentration of 15%), showed that none of the hydroxyproline and less than 3% of the total radioactivities of H^3hydroxyproline and H^3-proline could be recovered as protein-bound amino acids. Further chromatography of the deproteinized media on the Dowex 50-H^+ resin, as described, revealed that approximately 65% to 75% of the hydroxyproline and proline were present as free amino acids. Incubation of purified, reconstituted collagen fibrils, or gelating solutions prepared from the fibrils, with tissue culture media and parathyroid extract under identical conditions used for the calvaria, showed that the collagen fibrils were not solubilized and no free hydroxyproline was

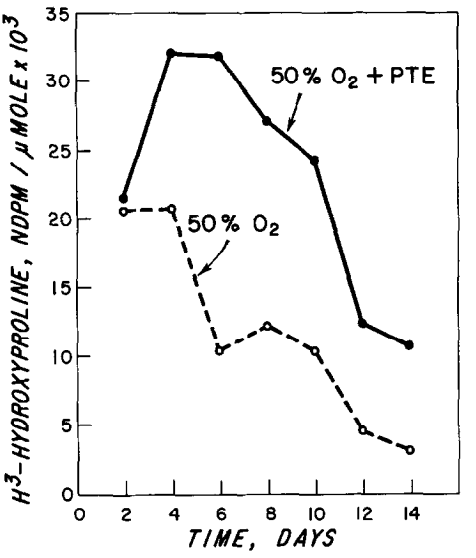

Fig. 3.--The specific activities of the total hydroxyproline recovered in tissue culture media during the active resorption of bone.

released. Therefore, the finding of peptide-bound and free hydroxyproline and proline in the tissue culture media during bone resorption suggests that the collagen is enzymatically degraded in the bone tissue, rather than being released as either soluble collagen or gelatin. Further studies are underway in an effort to elucidate the enzyme or enzymes involved in the degradation of collagen during bone resorption.

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