Parathyroid Influence on Removal of Hydroxyproline by Peritoneal Lavage in the Rat.* (28995)

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Recent studies reported from this laboratory demonstrated that levels of hydroxyproline in rat plasma were consistently lowered by parathyroidectomy(1). The source of this parathyroid-dependent hydroxyproline was presumed to be collagen, and the data presented were consistent with the hypothesis that, along with calcium, collagen was removed from bone under parathyroid influence. These results suggested that under normal conditions this protein was degraded to a form detectable without hydrolysis(1), while under conditions of intense parathyroid stimulation such as nephrectomy(2) the increase in circulating hydroxyproline was notable only after hydrolysis. The latter results suggested that under increased parathyroid activity hydroxyproline residues were transferred to plasma in a bound form.

The studies included in this report were directed toward determining the levels of hydroxyproline diffusible into the extracellular fluid under different levels of parathyroid function. Utilizing the technique of peritoneal lavage described by Talmage *et al*(3), the hydroxyproline which diffused into lavage fluid during each hour of equilibration was compared with diffusible calcium as an established reference of bone resorption(3). Subsequently, samples of plasma were subjected to gel filtration in order to obtain preliminary data as to the size of the molecule which contained the hydroxyproline detectable only after hydrolysis.

Materials and methods. The animals used in these studies were male Holtzman rats weighing 180 to 220 g. Animals were maintained on a calcium-free diet for 3 days before use. The lavage procedure followed that reported previously by Talmage *et al*(3). In this procedure 30 ml of fluid was introduced into the peritoneal cavity and allowed to equilibrate for one hour, after which time it was removed and replaced with fresh fluid. This was continued hourly until termination of the experiment. The lavage rinse used was the isotonic buffered solution previously described(3). For studies with added calcium, calcium lactate was added to the lavage fluid to give 10 mg of calcium per 100 ml of solution, and the lactic acid of the fluid was decreased by an amount equal to the added lactate ion. Blood was taken by heart puncture from animals under ether anesthesia, and the plasma was centrifuged and stored as described previously(1).

The ninhydrin procedure used for total amino acids was that of Troll and Cannan (4). Samples were analyzed for hydroxyproline by a modification of the method of Prockop and Udenfriend(5). The ion-exchange on Dowex-50 as described was derived from the procedure of Partridge and Elsden (6). Plasma samples were fractionated by gel filtration on Sephadex as reported recently(7,8).

Results. A. Lavage studies: The levels of hydroxyproline removed in the lavage fluid from all groups of animals are shown in Table I. The data are divided into 2 groups: The first includes the values from those animals receiving a calcium-free peritoneal rinse, thereby producing a marked loss of calcium from the animals; the second group includes values from rats receiving sufficient calcium by means of the rinse to produce a slight positive retention of calcium. In the first group, endogenous parathyroid secretion is stimulated; in the second group it is depressed(9). The calcium values have been omitted as they follow the pattern reported previously(3,9).

From the data in Table I, several observations can be made which relate changes in

* This work was supported in part by grants from A.E.C. and N.I.H.

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TABLE 1. Removal of Free Hydroxyproline by Peritoneal Lavage.

		Avg hours 1-4	Avg hours 5-8	Avg hours 9-12	Avg hours 19, 21, 23
Buffered calcium free rinse	1. Intact	2.4 ±.06 (59)	2.3 ±.05 (59)	2.0 ±.06 (28)	2.0 ±.11 (14)
	2. PTX	2.2 ±.06 (76)	*2.0 ±.06 (76)	*1.5 ±.06 (30)	*1.5 ±.06 (15)
	3. NEPHX	2.6 ±.07 (48)	*2.6 ±.09 (48)	*2.7 ±.10 (35)	
	4. NEPHX-PTX	*1.7 ±.08 (37)	*1.7 ±.08 (31)	*1.6 ±.10 (23)	
Rinse containing calcium, 10 mg/100 ml	5. Intact	2.1 ±.16 (12)	*1.7 ±.15 (12)		*1.2 ±.09 (20)
	6. PTX	2.2 ±.14 (12)	*1.8 ±.10 (12)		
	7. NEPHX	2.1 ±.13 (20)	*1.9 ±.11 (15)	†2.0 ±.08 (15)	

All values expressed as μmoles $\times 10^{-2} \pm$ S.E.
No. in parentheses = total No. of lavage values for a 4-hr interval.
PTX = parathyroidectomized.
NEPHX = nephrectomized.
* Differ from group 1 intact with $P < .001$. † Differ from group 3 NEPHX with $P < .001$.
Lavage values for all animals for a 4-hr period were averaged to obtain a single mean. S.E. is, therefore, based on the deviation of all values about this mean and N = number of lavage values for a 4-hr interval.

the imino acid removal both to calcium removal and to the state of endogenous parathyroid secretion:

(1) When a calcium-free rinse was employed, parathyroidectomy consistently reduced the amount of imino acid removed in the lavage fluid. While not as consistent as was the effect on the drop in calcium removal, the reduction in the removal of hydroxyproline was in the same range (25% *vs* 35%).

(2) Nephrectomy increased the amount of hydroxyproline removed by lavage. This is discussed further below.

(3) In the control animals, administration of calcium in the peritoneal rinse reduced the amount of hydroxyproline removed to the parathyroidectomized level.

(4) In the nephrectomized rat, administration of calcium reduced the amount of imino acid removed, but to a level significantly above the parathyroidectomized level by the ninth hour of lavage.

Values for total hydroxyproline after hydrolysis were also obtained for these lavage samples. Hydrolysis did not increase the imino acid values obtained for lavage wash removed from animals with intact kidneys. However, for nephrectomized rats, all hydrolyzed values were slightly higher than the nonhydrolyzed value for the same sample, with parathyroidectomy still producing a significant drop. This is consistent with an increased production of a bound form of hydroxyproline in nephrectomized animals as was previously described(1) and may explain the divergent effect of the administration of calcium to nephrectomized animals in item 4 above.

B. Fractionation studies: Preliminary studies were carried out in an attempt to characterize the molecule or molecules which contained hydroxyproline in plasma. Plasma samples were fractionated by gel filtration on Sephadex G-25, which is known to exclude molecules larger than MW 3500-4500, dextran equivalent(7). A G-25 column 0.9 × 70 cm was calibrated by passing through it 1.0 ml samples of plasma from control and nephrectomized animals and eluting with 0.1 M KCl. Calibration was completed by analyzing the 1.0 ml fraction collected, both for total hydroxyproline and for total amino acids by ninhydrin following hydrolysis. The included and excluded peaks were separated by 4 fractions, allowing essentially complete separation.

Samples of 1.0 ml of plasma taken from control, parathyroidectomized, nephrectomized, and parathyroidectomized-nephrectomized animals were then separated on the gel column into 10 ml included and excluded fractions. These fractions were evaporated to dryness on a steam bath, then hydrolyzed in 6 N HCl in sealed tubes at 115°C for 18 hours. The hydrolysates were evaporated to dryness in a steam bath, and redissolved in 0.2 M potassium citrate buffer, X pH 2.5. Samples were then fractionated on Dowex-50

TABLE II. Fractionation of Plasma by Gel Filtration.

		Hydroxyproline values (Samples)			
		1	2	3	Avg
Excluded	NEPHX	10.7	10.7	5.3	8.9
	CONTR	3.9	3.3	3.7	3.6
	PTX-NEPHX	9.9	9.0	7.8	8.9
	PTX	2.8	2.1	2.5	2.5
Included	NEPHX	7.8	11.0	7.4	8.7
	CONTR	5.5	4.6	4.6	4.9
	PTX-NEPHX	4.3	—	4.4	4.4
	PTX	1.5	2.2	3.8	2.5

All values are in μmoles/ml of plasma $\times 10^{-2}$.
PTX = parathyroidectomized.
NEPHX = nephrectomized.

as previously described, and the fractions known to contain hydroxyproline were pooled and analyzed for this imino acid.

The results of the fractionation studies are summarized in Table II. Three samples were analyzed for each condition, and some variation in results was introduced by the combined column procedures. This highly specific combination of ion-exchange separation with a specific hydroxyproline method gave confirmation to previously reported increases in hydroxyproline of plasma following nephrectomy(1). Although this increase occurred in both the included and the excluded fractions, the increase seen in the excluded fraction was not offset by simultaneous parathyroidectomy. However, the increase in hydroxyproline in the included fraction was completely offset by simultaneous parathyroidectomy, and hence is parathyroid-dependent. Since there was no increase in free hydroxyproline in plasma of the nephrectomized animal(1), this parathyroid-dependent increase following nephrectomy must be of a bound form of hydroxyproline of low molecular weight, apparently less than MW 4500. Further studies must be carried out to confirm this finding.

Discussion. These lavage studies give further confirmation of the presence of free hydroxyproline in extracellular fluids which is diffusible and the production of which is influenced by parathyroid activity. This is shown both by direct comparisons of control and parathyroidectomized animals, and by the administration of physiological levels of calcium as a depressant of parathyroid activity.

The source of the parathyroid-dependent hydroxyproline would most likely be collagen of bone matrix. Hydroxyproline constitutes approximately 14% of the weight of collagen which must be degraded in the process of maintenance of calcium homeostasis in opposition to imposed calcium depletion(1). Plasma levels of this imino acid are extremely low(11), but other possible sources such as intermediates in collagen synthesis or collagen of other tissues cannot be ruled out. Also, the source of the hydroxyproline which remains in the plasma and extracellular fluid of the parathyroidectomized animal is difficult to identify. While calcium equilibrates at a basic level in the absence of parathyroid activity(9,10), an imino acid as a component of protein would not be subject to the same solubility effects.

Preliminary fractionation studies indicate that the hydroxyproline, present as a consequence of parathyroid activity, is contained in a small and presumable diffusible molecule. The increase in total hydroxyproline of plasma following nephrectomy (Table II) appears partly as a large molecule which is independent of parathyroid activity, and in part, as a smaller molecule which is present as a result of parathyroid mediation.

Analysis of plasma following 2 fractionation procedures which reduces the possibility of interference with the analytical procedure by products of hydrolysis, also confirmed an increase in bound hydroxyproline of plasma following nephrectomy.

Summary. Utilizing the technique of peritoneal lavage, levels of hydroxyproline and calcium in lavage fluid and plasma were studied in intact and parathyroidectomized rats with and without concomitant nephrectomy. A limited characterization of the molecule containing this imino acid also was made by gel filtration. Levels of free hydroxyproline removed from parathyroidectomized rats were consistently lower than from intact rats. These differences were greatly magnified by

concomitant nephrectomy. However, the endogenous parathyroid stimulation produced by nephrectomy also resulted in an increase in bound hydroxyproline which is detectable only after hydrolysis of the lavage fluid or plasma. The results indicate that hydroxyproline levels in plasma and lavage fluid are parathyroid-dependent and can be correlated with calcium removal under the conditions of parathyroidectomy and/or nephrectomy. These studies further suggest that the breakdown of bone under the influence of parathyroid hormone includes both its organic and inorganic components.

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Received October 22, 1963. P.S.E.B.M., 1964, v115.

Effect of Zinc on Cancerogenesis by Cadmium.* (28996)

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Previous experimental reports have brought out that in some species the metals chromium (1,2), cobalt(1), gold(3), nickel(1,2,4,5), platinum(3), silver(2,3) and tantalum(2) are capable of inducing tumors when introduced into femur, pleura, nasal sinus, intramuscular or subcutaneous tissue sites. Heath (6-8) also investigated the cancerogenic properties of cobalt and found that the metal powder, suspended in fowl serum, produced rhabdomyosarcomas when injected intramuscularly into rats. Metabolic studies had revealed that one of the effects of cobalt was inhibition of the enzymatic oxidation of *a*-ketoglutarate(9). Since cadmium was also known to be an inhibitor of keto-acid oxidation(10), Heath and co-workers(11) investigated whether cadmium, like cobalt, was also cancerogenic. From 14 to 28 mg of a powdered form of cadmium metal, suspended in fowl serum, was injected intramuscularly in rats in a similar manner as in the studies with cobalt. These investigators did, indeed, find that cadmium produced tumors, chiefly rhabdomyosarcomas, at the site of injection. Zinc and tungsten, similarly administered were not tumorigenic(7).

In recent years there has been renewed interest in cadmium, particularly since Parizek and Zahor(12) called attention to the fact that subcutaneously administered cadmium caused destruction of the rat and mouse testis and that these acute effects could be prevented by simultaneous administration of large amounts of zinc. These observations have since been confirmed by others(13-16). A recent report from this laboratory(17) brought out that, in both rats and mice, interstitial cell tumors of testis resulted from a single injection of cadmium chloride (0.03 mmole/kg) administered subcutaneously in the interscapular region and that this tumor formation could be prevented by simultaneous administration of zinc acetate (3.0 mmole/kg).

* Supported in part by a U. S. Public Health Service research grant from Nat. Cancer Inst., and in part by a research grant from U. S. Atomic Energy Commission.