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Thyrocalcitonin Suppression of Hydroxyproline Release from Bone*† (32629)

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It is now generally accepted that the hypocalcemic effect of thyrocalcitonin is mediated primarily by a reduction in the rate of calcium removal from bone (1–5). However, as is still true with parathyroid hormone, the actual mechanism by which this recently discovered hormone accomplishes its effect is still to be elucidated. In most aspects studied, thyrocalcitonin has been shown to be antagonistic to parathyroid hormone, but its action is independent as it is fully effective in parathyroidectomized animals (6). A relevant current controversy concerning parathyroid hormone action on bone is related to its ability to increase the resorption of all components of bone: apatite crystal, collagen and the mucopolysaccharide ground substance. One can not say with certainty whether the hormone affects all intercellular bone components concurrently, or whether the action on one component is primary and those on the other components are produced indirectly.

At the present time, thyrocalcitonin action on bone has been studied briefly in regard to its ability to decrease the transfer of calcium and phosphate from bone to extracellular fluid. It is the purpose of this report to examine its effect on collagen breakdown and to determine whether thyrocalcitonin is antagonistic to the action of parathyroid hormone on this process. Friedman and Raisz (7), Gaillard (8) and Aliapoulios *et al.* (9) have reported that, in *in vitro* systems, thyrocalcitonin can block effects normally produced by

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parathyroid hormone on total bone resorption. It was therefore of interest to determine whether the effects of the new hormone were antagonistic to those of parathyroid hormone on the breakdown of organic matrix of bone in an *in vivo* system, as measured by extracellular hydroxyproline levels. Extracellular hydroxyproline has been shown to be a reliable index of collagen brreakdown (10–12). The studies to be presented examine the effect of thyrocalcitonin on bone matrix catabolism under the following experimental conditions which have been shown to alter parathyroid hormone stimulated bone resorption: parathyroidectomy, calcium restricted diet, nephrectomy, and peritoneal lavage (13–17).

Materials and Methods. Animals and diets. Male Holtzman rats (200–220 gm) were used in all experiments. Animals were maintained on stock diet and transferred to a calcium free diet (CFD) (13) at times indicated.

Experimental procedures. All surgery was carried out under light ether anesthesia. Parathyroidectomy (PTX) was performed 9 hours before the start of the lavage procedure. Bilateral nephrectomy (NEPHX) was accomplished through a single midventral incision.

The technique of peritoneal lavage, with a calcium and phosphate free, isotonic and buffered rinse was employed as has been described in previous publications from the laboratory (15).

Analyses. Calcium values were determined by an automated fluorometric procedure designed for the Auto Analyzer (18). Inorganic phosphate was measured by the method of Allport and Keyser (19). Hydroxyproline was quantitatively measured by the method of Prockop and Udenfriend (20) as modified by Bates *et al.* (13).

Thyrocalcitonin preparation. Partially purified extracts of hog thyroid were prepared according to the methods of Hirsch *et al.* (6). The resulting powder dissolved in a 0.05 *M* acetate buffer at pH 3.8 was used experimentally. Bioactivity of this extract was determined by bioassay (6). The preparation used in these studies had activity of 1400 Hirsch units/mg N (21).

Experimental designs. Two series of experiments were carried out.

Series I. Animals were placed on CFD 18 hours before the start of an 8-hour peritoneal lavage. Half of the animals were parathyroidectomized. Thyrocalcitonin (TC) (8–16 Hirsch units) was injected subcutaneously three times: 15 min before the start of the lavage, and after the second and fifth hours. Only free hydroxyproline values for nonhydrolyzed lavage fluid were determined, since a previous report (14) had shown that lavage fluid contained little or no peptide bound hydroxyproline.

Series II. Normal and nephrectomized (NEPHX) experimental animals were used. These animals were maintained on CFD and deionized water for 3 days prior to sacrifice. Thyrocalcitonin (8–16 Hirsch units) was administered every 2 hours for a 24-hour period to half of each group. In addition, a single control group maintained on stock diet was also sacrificed. Blood was obtained by cardiac puncture and serum obtained from the blood was analyzed for hydroxyproline after hydrolysis in 6 *N* HCl for 18 hours according to the procedure of Bates *et al.* (13).

Results. Series I: Effects on hydroxyproline removed by peritoneal lavage. As is shown by the data summarized in Fig. 1, repeated administration of thyrocalcitonin to animals undergoing peritoneal lavage dramatically reduced the removal of calcium and phosphate, in agreement with past studies from this laboratory (2). Of special importance to this study however, is the observation that in both intact and parathyroidectomized animals, thyrocalcitonin eventually also reduced the amount of free hydroxyproline removed by the lavage procedure. Although calcium and phosphate removal rates were statistically reduced within 2 hours, only after the fourth or fifth hour of lavage did a statistically significant reduction occur in the hydroxyproline levels in lavage fluid or thyrocalcitonin treated animals.

The cumulative effect of thyrocalcitonin administration was determined from the sum of hourly values (Table I). In both control and parathyroidectomized rats, thyrocalcitonin caused a 20% reduction in the total amount of hydroxyproline removed during the 8-hour lavage. In addition, a similar drop in removal rate of hydroxyproline caused by parathy-

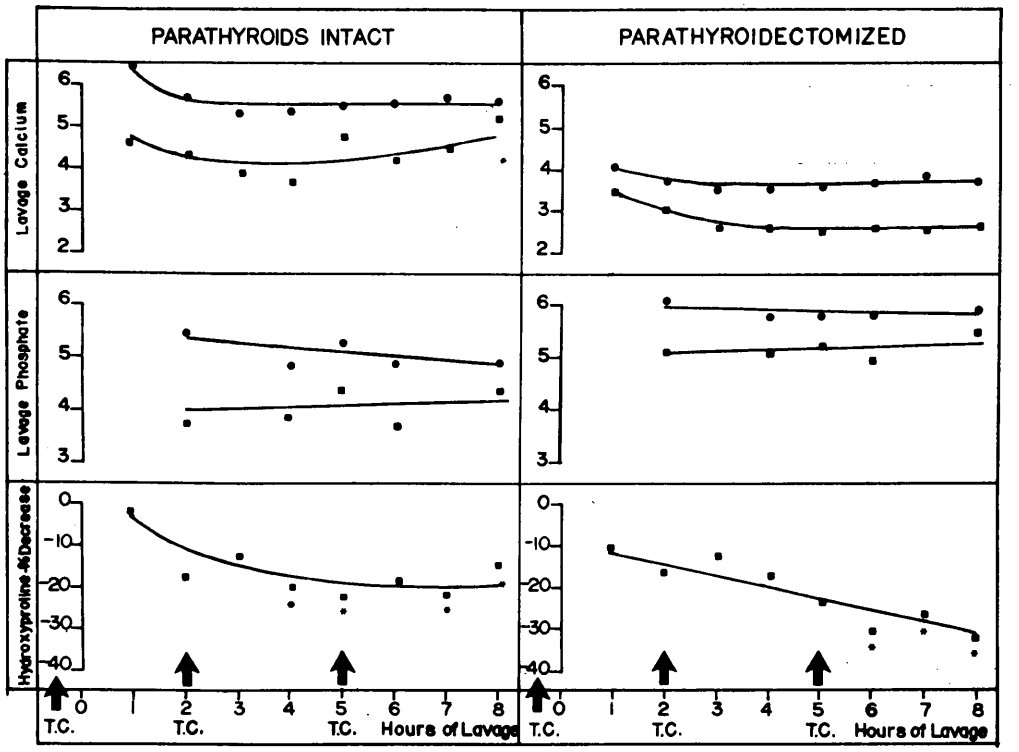

FIG. 1. Effects of repeated thyrocalcitonin administration during peritoneal lavage. ● without thyrocalcitonin; ■ with thyrocalcitonin.

Notes: (a) All points represent average of 10 or more values. (b) Calcium and phosphate values for TC treated animals are significantly different from control values with a $p < .001$; and (c) Hydroxyproline values are presented as percentage change from their respective controls. For starred hours, the drop was statistically significant with a $p < .01$.

roidectomy alone was observed, confirming previous findings (14).

Series II: Comparison of thyrocalcitonin effects in control and nephrectomized rats. In agreement with past studies (13), calcium deprivation and/or nephrectomy produced an increase in hydrolyzed serum hydroxyproline (Table II) when compared to animals on stock diet. Administration of thyrocalcitonin not only prevented this increase, but reduced the hydroxyproline values to lower levels than observed in the control group.

Discussion. These experiments demonstrate that thyrocalcitonin administration is capable of suppressing the levels of extracellular hydroxyproline, even in situations where this parameter is usually elevated. Since this imino acid has been shown to be a good indicator of collagen degradation, it can be concluded that thyrocalcitonin inhibits this process. It can be inferred also that this inhibition of collagen catabolism occurs in the organic matrix of bone, since 50% of collagen is in bone, and because of the concurrent striking effects observed on bone mineral removal.

Semiquantitative approximations of the amount of bone, the catabolism of which is being prevented by thyrocalcitonin, provide an interesting approach to the question of whether there are equal effects on bone mineral and organic matrix. Lavage data indicate that thyrocalcitonin prevented the removal of 2.6 mg of calcium and of 0.1 mg of free hydroxyproline during the 8-hour lavage period in intact animals. Assuming 25% of dry bone is calcium (22) this indicates that 10 mg of bone were not degraded as the result of thyrocalcitonin treatment. Because free hydroxyproline represents only 30% of the total hydroxyproline arising from collagen degradation (13) the value of 0.1 mg for free hydroxyproline is corrected to 0.3 mg.

TABLE I. Effect of Thyrocalcitonin on Total Hydroxyproline Removed by 8-Hour Peritoneal Lavage.

Group	μmoles of HO Pro/8 Hours ± SE
Intact animals	
Intact controls (12)	5.56 ± .16
Intact thyrocalcitonin (12)	4.70 ± .22
Difference	0.86 ± .27 $p \leqq .005$
Parathyroidectomized animals	
Parathyroidectomized controls (11)	4.51 ± .16
Parathyroidectomized thyrocalcitonin (10)	3.51 ± .21
Difference	1.00 ± .28 $p \leqq .005$

TABLE II. Effect of Thyrocalcitonin on Hydroxyproline Values of Hydrolyzed Serum.

Group	HO Pro (μmoles/ml + SE)	% of control
Control (6)	.27 ± .01	100
CFD (6)	.28 ± .01	106
CFD + TC (6)	.24 ± .005	90
Difference	.04 ± .008 $p \leqq .001$	
NEPHX + CFD (6)	.31 ± .01	118
NEPHX + CFD + TC	.24 ± .01	91
Difference	.07 ± .01 $p \leqq .005$	

Assuming hydroxyproline constitutes 4% of dry bone,[2] it can be estimated that thyrocalcitonin administration prevented the removal of 8 mg of bone. These two approximations of the suppression of total bone removal are in surprisingly good agreement, and support the hypothesis that thyrocalcitonin reduced equally the process of bone mineral removal and organic matrix degradation.

However, in the lavage studies, it was noted that the appearance of a reduction in hydroxyproline removal rates occurred considerably later than that for the removal of calcium and phosphate. In fact, the effect on the imino acid can be shown only if repeated doses of thyrocalcitonin are used.[3] It might be concluded from this that the effect on collagen was secondary to a primary effect on bone salt removal. An alternative explanation could be that the delay was observed due to the increased time necessary for the complete hydrolysis of collagen and the transport of the hydroxyproline to the body fluids.

From the findings just discussed and the demonstration that thyrocalcitonin can substantially reduce serum hydroxyproline levels in nephrectomized animals, it seems probable that thyrocalcitonin is antagonistic to parathyroid hormone in regard to the latter's ability to stimulate the removal of inorganic salts and organic matrix from bone. This *in vivo* study supports the findings of Friedman *et al.* (7), Gaillard (8) and Aliapoulis *et al.* (9) as to thyrocalcitonin's ability to abolish these effects of parathyroid hormone. Also, the findings of MacIntyre *et al.* (25) and Pechet *et al.* (26) who reported that thyrocalcitonin reduced the levels of urinary hydroxyproline are in agreement with these studies.

It seems evident from these studies that exogenous thyrocalcitonin does inhibit total bone resorption when given over an extended period of time.

Summary. The effects of thyrocalcitonin on extracellular levels of hydroxyproline were examined in the following situations which normally alter this parameter through effects on bone resorption: peritoneal lavage, parathyroidectomy, nephrectomy, and calcium restricted diet. It was found that thyrocalcitonin reduced extracellular hydroxyproline levels in all situations studied. Effects on removal of hydroxyproline and calcium by peritoneal lavage were of such magnitude to suggest that the entire process of whole bone catabolism is inhibited by thyrocalcitonin. Marked effects of thyrocalcitonin in the presence of increased levels of endogenous parathyroid hormone indicate that thyrocalcitonin can block the effects of parathyroid hormone. It is concluded

[2] This approximation is based on hydroxyproline constituting 14% of collagen (23), collagen constituting 90-96% of organic matrix and organic matrix constituting 30-35% of bone (24).

[3] Klein, D. C. and Talmage, R. V., unpublished results, 1967.

that repeated administration of thyrocalcitonin results in an inhibition of all phases of bone resorption.

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The Effect of Bile on Vitamin A Absorption in the Rat* (32630)

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It is generally accepted that after absorption long chain fatty acids are mainly converted to triglyceride in the intestinal mucosa and then leave the intestine by the lymphatic route (1, 2). Recent evidence suggests that in the absence of bile the partition of long chain fatty acids between the portal vein and lymph is altered to favor the portal route (3, 4) and this route may be used to a variable degree normally (5). We have now extended these observations using vitamin A as another model compound, for it is usually considered that absorbed vitamin A is esterified and also transported by the lymphatic route (6).

Material and Methods. Crystalline unlabeled vitamin A acetate, palmitate, and alcohol were a gift from Roche Products Limited, Basle. Standard solutions were prepared in *n*-hexane. Vitamin A acetate (Carbinol ^{14}C) (Radiochemical Centre, Amersham, England) had a specific activity of 2.92 mC/mole. The radiochemical purity by thin-layer chromatography (TLC) on silica gel G (cy-

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