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Effect of Heparin and Parathyroid Extract on Acid Phosphatase In Bone.* (30963)

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It is generally agreed that normal remodelling of bone is a cellular process taking place in well defined areas influenced by growth and mechanical stress(1,2). The mechanism of the process is less well understood, but osteoclasts and giant cells are usually to be found in resorptive cavities in the area being remodelled(3,4). Various hormones have been shown to alter the normal process when given in pharmacologic amounts(1). Parathormone is used experimentally to increase resorption presumably by accelerating normal processes (1,5,6). After treatment of an animal with parthyroid extract (PTE) changes can be shown in both the stable highly mineralized diaphyseal region, and in the more cellular metabolically active metaphyseal regions. These changes may reflect different independent parameters of parathyroid action(7-10).

Since the early work of Gutman, Huggins, Kochakian, and Klendshoj, parathyroid induced resorption or pathologic destruction of bone in metastatic cancer has been associated with elevated serum acid phosphatase and elevated bone acid phosphatase(11-15). In more recent studies various workers have associated acid phosphatase with osteoclasts in PTE-treated and normal bone by histochemical means(16-21).

Acid phosphatase as well as collagenase and hydrolases are found in many tissues in subcellular particles, the lysosomal bodies of de Duve(22). De Duve has suggested that release of these enzymes from lysosomal bodies of osteoclasts may help to explain the local action of osteoclasts on bone(23). It has been postulated that in order for this to happen the lysosomal membrane or cell membrane must be altered to permit escape of the enzymes(24). Experimental evidence for this has been presented by Asher and Nichols, studying heparin induced osteoporosis, who found that heparin could cause a marked increase in collagenase activity in bone. These same authors also reported a similar action for saponin and chondroitin sulfate(25). Another lysosomal membrane active substance, vitamin A, has been shown to enhance resorption of bone in tissue culture(26). These considerations have become clinically important with the discovery of osteoporosis in patients on long term heparin therapy(27,28). Increased serum levels of acid phosphatase have been reported in hyperparathyroid states(11-14). It appears that the clinical observations of Griffith *et al*(27) might be explained on the basis of a still further liberation of acid phosphatase resulting from heparin activity. To test this hypothesis we injected heparin into animals made hyperparathyroid with PTE and analyzed the bone for acid phosphatase in areas known to be subject to PTE ac-

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tion. The results reported here are in accord with this hypothesis.

Materials and methods. Male Wistar rats 32 days of age, average weight 78 g, were fed a natural stock ration containing adequate vitamin D.§ They were subcutaneously injected 7 hours prior to sacrifice with 1 unit parathyroid extract‖ per gram body weight. They were also injected with "3 units" per gram body weight of a crude preparation of PTE¶ 6 hours prior to sacrifice. The dosage used was in the similar pharmacologic range to that used by Young in studying bone resorption(1). The PTE extract administered was so chosen that a 2 mg% increase in serum calcium was obtained as discussed by Rasmussen(29). This was necessary in order to check on the pharmacologic potency of the extract. The 6-hour duration of treatment was chosen as giving the peak activity as judged by serum calcium elevation according to Jeffay and Bayne(10). No adverse effect of this dosage was observed in the 6-hour period of observation in comparison with controls. Heparin (3 mg/100 g body wt) was given subcutaneously ½ hour prior to sacrifice. The heparin dosage was twice that used for anticoagulation in the rat and given ½ hour prior to sacrifice to insure absorption following subcutaneous injection(30).

The efficacy of the PTE was determined by measuring the serum calcium(31), while the prolonged clotting time was used as a criterion for the heparin effect.

Animals were anesthetized with ether, blood was drawn by cardiac puncture, and the animals dispatched with an overdose of ether. Dissection was done rapidly and animals were stored in the cold (4°C) until dissection could be completed (within 1½ hrs). Bone was dissected free of all soft tissue, separated into calvaria, metaphysis and diaphysis, wiped free of marrow and frozen overnight.

Bone powder was prepared by placing frozen bone in stainless steel capsules, in powdered dry ice. While frozen, each kind of bone was powdered separately in a Wig-L-Bug (dental amalgam machine for preparing tooth fillings). Care was taken to prevent increase in temperature during this process. The powder was then transferred to a weighed test tube, reweighed and stored in cracked ice until all samples were powdered. Sample size was 50 to 100 mg.

Acid phosphatase was determined by the method of Fujita(32) as modified by Sigma Co.(15). Substrate (p-nitrophenyl phosphate) plus citrate buffer pH 4.8 (total volume 1.2 ml) warmed to 38°C was added to the bone and it was mixed with a Vortex Mixer. It was then incubated at 38°C for 30 minutes. The reaction was stopped with 1 ml 10% TCA and transferred quantitatively to centrifuge tubes using 3 ml distilled water. One ml 1.1 N NaOH was added with mixing, to develop the color and the tubes were centrifuged for 15 minutes at maximum speed on an International Centrifuge. Optical density at 410 mμ was read on a Coleman Jr. Spectrophotometer and results were expressed as Sigma Units per gram wet weight bone.

Results. Acid phosphatase activity in the 3 kinds of bone studied differed from one another. In the calvaria, Table I, both PTE alone and heparin alone increased the acid phosphatase activity a small but statistically insignificant amount. However, the effect of PTE and heparin together increased the acid phosphatase to a highly significant degree.

In the metaphysis (Table II) again both heparin and PTE alone enhanced the acid phosphatase activity, heparin only slightly

TABLE I. Acid Phosphatase in Rat Calvaria. Sigma units per gram wet weight bone.

Group	No. of animals	SU/g ± S.E.*	p value†
Control	15	131.7 ± 4.7	
PTE	10	147.5 ±10.2	<.5
Heparin	10	149.9 ± 9.6	<.5
PTE and heparin	5	235.5 ± 3.4	<.001

$$* \text{S.E.} = \pm\sqrt{\frac{\Sigma d^2}{n(n-1)}}.$$

$$\dagger \text{S.E.}_{M_1-M_2} = \sqrt{(\text{S.E.}_{M_1})^2 + (\text{S.E.}_{M_2})^2}.$$

$$t = \frac{M_1 - M_2}{\text{S.E.}_{M_1-M_2}}.$$

§ Purina Laboratory Chow.

‖ Injection parathyroid USP 100 units per cc.

¶ Gift of Dr. M. M. McGuire of Eli Lilly Co., gratefully acknowledged.

TABLE II. Acid Phosphatase in Rat Metaphyses. Sigma units per gram wet weight bone.

Group	No. of animals	SU/g ± S.E.	p values
Control	10	648.3 ± 27.6	
PTE	10	936.0 ± 98.7	<.05
Heparin	10	724.2 ± 22.2	<.1
PTE and heparin	5	1084.9 ± 91.7	<.001

while PTE was significant at the $p<.05$ level. On the other hand, the combination of PTE and heparin was very effective in elevating acid phosphatase activity ($p<.001$).

On the contrary, the diaphysis (Table III), showed no significant change in acid phosphatase activity after any of the treatments.

Table IV shows blood calcium levels of control and PTE-treated animals. PTE caused a 2 mg/100 ml elevation in serum Ca^{++} level.

Discussion. The results observed here are suggestive of a synergistic action between PTE and heparin on acid phosphatase activity. The calvaria, a membranous bone, and "chemically the tissue intermediate between soft tissue and mature bone" according to Dowse *et al*(33) showed intermediate activity between diaphysis and metaphysis. This is probably related to the highly heterogeneous cell population consisting of osteoblasts, osteocytes, osteoclasts, cartilage cells and preosseous mesenchymal cells. The highly significant effect of heparin on acid phosphatase activity could result from a releasing effect of heparin on enzymes present in increased amounts within cells following PTE treatment. It may be that the proportion of cells containing increased amounts of acid phosphatase is so small that the effect is not seen until the enzyme is released. Goldhaber found that suboptimal amounts of vit A, a membrane active agent, or PTE alone did not cause resorption of calvaria in tissue culture until the two were combined(26).

TABLE III. Acid Phosphatase in Rat Diaphyses. Sigma units per gram wet weight bone.

Group	No. of animals	SU/g ± S.E.	p values
Control	15	60.7 ± 9.5	
PTE	10	76.5 ± 14.8	<.5
Heparin	10	75.4 ± 4.8	<.5
PTE and heparin	5	74.4 ± 13.8	<.5

TABLE IV. Serum Drawn at Time of Sacrifice.

Group	No. of animals	Avg Ca^{++} (mg/100 ml)
Control	15	9.7
PTE	10	12.2
PTE and heparin	5	12.4

In this connection, it might be noted that the acid phosphatase content of the osteoclasts in the "ia" rat, a mutant strain characterized by severe impairment of physiologic bone resorption, was found to be at least twice as active as normal rats. Handelman *et al* postulated that there might be an abnormality of the cell membrane preventing the contained enzymes from reaching the underlying bone(19). It is of interest that this defect can be overcome by early administration of PTE(34).

The lack of effect of the treatment in the diaphyseal region of bone is interesting. This could be explained by the relatively small number of osteoclasts present in proportion to the mass of matrix so that any effect is masked by the large amount of inert material. Talmage and others have shown rather marked metabolic differences between activity of the diaphysis *vs* the metaphysis(1,8, 35). The present observations are further evidence of this metabolic difference.

The metaphysis, on the other hand, showed some effect even from heparin alone, when compared with controls, perhaps a release of the endogenous acid phosphatase present in normal cells. Likewise the dose of PTE used alone caused a significant elevation of bone acid phosphatase. This is in agreement with studies by other workers(11,13). The most dramatic effect was caused by the combined treatment with both heparin and PTE in the metaphysis, the portion of bone concerned with remodelling and known to be sensitive to parathyroid hormone. This result supports the hypothesis that acid phosphatase can be released by heparin in areas of active bone resorption although it in no way proves or disproves the presence of lysosomes in osteoclasts.

In view of these recent findings the prolonged clinical use of heparin in elderly pa-

tients or orthopedic patients should be re-evaluated.

Summary. To test the theory that heparin might stimulate acid phosphatase in the bone of young rats, heparin, PTE, or a combination of both were injected. After an appropriate time interval acid phosphatase activity of bone of treated animals was compared with controls. It was found that heparin enhanced the PTE stimulated acid phosphatase activity of both calvaria and metaphysis but not diaphysis. The possible relation of these facts to the presence of lysosomes in osteoclasts was discussed.

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