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The Effect of Parathyroid Hormone upon Release of Degradative Enzymes from Rat Bone (40642)[1]

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Parathyroid hormone action on bone involves the mobilization of both calcium and phosphate and the resorption of organic matrix (1, 2). Many of the biochemical events which accompany these two effects of the hormone are recognized (3–5) but their time sequence is unclear and the role of some of the components of normal bone matrix has not been elucidated. Specifically, lipids have been recognized as being part of the normal chemical makeup of bone matrix for some time (6, 7). While some progress has been made in understanding the association of these lipids with bone formation (8) and mineral transformation (9), there still appears to be a question as to their function. In an attempt to add information with regard to this question, the following series of experiments were carried out.

Materials and methods. All experiments were carried out on specimens from 10-day-old male Wistar rats (body wt 19.5 ± 1.2 g).

Determination of phospholipids. In order to ascertain which phospholipids were degraded by parathyroid hormone, the following procedures were carried out:

Groups of five animals were sacrificed by decapitation. The tibia were removed immediately after sacrifice and were dissected free of soft tissue and periosteum. The bone marrow was removed by flushing with cold saline and the metaphyseal portions were separated and pooled. The pooled metaphyses of the distal femora and proximal tibiae were incubated in 5 ml of modified BGJB medium containing 2 units of purified bovine parathyroid hormone (Eli Lilly Co.) per milliliter in a Dubnoff incubator under 95% oxygen, 5% CO_2 for 4 hr. A control group without parathyroid hormone was incubated simultaneously. After incubation, the bones were washed with distilled water three times and lyophilized. The lipids were extracted according to the method of Folch *et al.* (10). The total lipid phosphorous of the lyophilized bone was determined according to the method of Marinetti (11) and the phospholipid content was calculated by multiplying the phosphorous content of the lipid extract by 25 (11). Individual phosphatides were determined by thin-layer chromatography separation. Thin-layer plates were prepared from silica gel H (Sigma Chemical Co.) and stored in a desiccator over anhydrous $CaSO_4$. The plates were activated at 100° immediately before use. Chromatograms were developed with chloroform–methanol–acetic acid–water (24:14:4:2 by volume) (12). After development, the entire area was exposed to iodine vapor to visualize the separated lipids and the phosphatides were scraped from the thin-layer plate. There were three extractions of the phospholipids with a mixture of ethanol/chloroform/water/acetic acid (100:30:20:2). The lipid phosphorus was determined according to the method of Marinetti (11). Because phosphatidyl choline showed a larger percentage loss than phosphatidyl ethanolamine or sphingomyelin (see Results), it was selected as the substrate for enzyme assays.

Enzyme activity. The metaphyseal portion of one tibia was incubated in 1.0 ml of modified BGJB medium containing 2 units of parathyroid extract and 0.05 ml of ether solution containing labeled phosphatidyl choline ([*methyl*-^{14}C]choline) (New England Nuclear, Boston, Mass.) and 1 mg of unlabeled phosphatidyl choline purified from commercially available egg lecithin. Control values were obtained by incubation without parathyroid hormone. Incubations were carried out at 37° with continuous shaking for times varying between 2 and 48 hr. At the end of the reaction, the lipids in the medium were extracted with chloroform/methanol three times. After extraction, the aqueous layer was centrifuged, following which 0.2 ml of the

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of the aqueous solution was counted in a liquid scintillation counter.

Collagenolytic assay. Collagenolytic activity was assayed by measuring the release of soluble radioactivity from purified reconstituted collagen labeled with [^{3}H]proline and [^{3}H]hydroxyproline according to the method of Kaufman *et al.* (14). The reaction mixture consisted of 100 μl of the ^{3}H-labeled collagen gel formed by warming to 37° for 20 hr (approx 4000 cpm) in 400 μl of a modified BGJB medium containing 2 units of parathyroid extract and one tibial metaphysis from a 10-day-old rat. The mixtures were incubated for periods varying from 2 to 48 hr at 37°. Control values were determined by incubation without hormone. Blank values were obtained by parallel incubation using heat-killed tibial metaphysis. After completion of the incubation, the cultures were centrifuged at room temperature, a 200-μl aliquot of supernatant of each culture was dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and counted in a liquid scintillation counter.

Determination of ^{45}Ca release. Six animals were injected subcutaneously with 2 μCi of ^{45}Ca. The animals were sacrificed by decapitation 20 hr later and the tibial metaphyses prelabeled with ^{45}Ca were dissected free of soft tissues and placed in 1.0 ml of a modified BGJB medium in pairs. One tibia was placed in control medium while the opposite tibia of the same rat was in medium supplemented with 2 units of purified parathyroid extract. Both plates were incubated at 37° at times varying from 2 to 48 hr after which 0.1 ml of the medium was dissolved in 10 ml of Aquasol (New England Nuclear, Boston, Mass.) and the radioactivity was determined in a liquid scintillation counter. Statistical analysis of results was carried out using Student's *t* test.

Results. As can be seen in Fig. 1, there was a statistically significant increase in the release of ^{45}Ca into the medium by bones incubated with PTH as compared to those without the hormone. This became statistically significant ($P < 0.05$) at 4 and 10 hr and remained significant ($P < 0.01$) at 24 and 48 hr. As is also seen in Fig. 1, there was a parallel increase in the radioactivity from [^{14}C]phosphatidyl choline with PTH again leading to an increase in the amount of radioactivity measured. This difference was statistically significant ($P < 0.01$) at 4 hr and remained so for the duration of the experiment. Figure 2 demonstrates that the degradation products from labeled collagen were not found until 5 hr after the administration of the hormone and parathyroid hormone did not appear to increase this until much later than calcium release had been noted and phospholipid breakdown had been measured. The differences were statistically significant at 24 hr ($P < 0.05$) and 48 hr ($P < 0.01$). Table I indicates that following 4 hr of incubation, a statistically significant decrease in total phospholipid was seen in bone. There was a statistically significant difference in the amount of phosphatidyl choline and while there was a diminution in phosphatidyl ethanolamine and sphingomyelin, this was not statistically significant.

Discussion. The sequence of events whereby parathyroid hormone mobilizes serum calcium and leads to matrix resorption is still not clear. The eventual outcome is

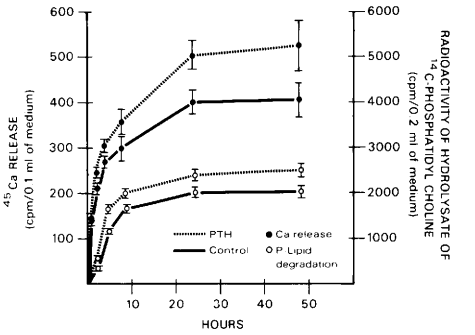

FIG. 1. The release of ^{45}Ca from prelabeled bones and of the degradation products from [^{14}C]phosphatidyl choline. Figures refer to time release and the standard error is shown. Each point is based on six determinations carried out in duplicate.

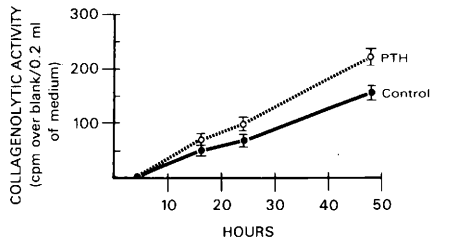

FIG. 2. The release of [^{14}C]proline and hydroxyproline from prelabeled collagen. Figures refer to time release and the standard error is shown. Each point is based on six determinations carried out in duplicate.

TABLE I. EFFECT OF PARATHYROID HORMONE ON PHOSPHOLIPIDS OF RAT BONE AFTER 4 HR OF INCUBATION[a]

	Total phospholipid	Phospholipids mg/g bone[b]		
		PC	PE	SM
Control	11.8 ±0.9	5.8 ±0.5	2.5 ±0.4	1.1 ±0.2
PTH (2 units/ml)	9.5* ±0.8	4.2* ±0.6	2.1 ±0.3	0.8 ±0.2

[a] Results are given as the mean ± SE of five determinations (five animals were used in one group and four metaphyseal bones used in one pooled sample). Significant differences between control and parathyroid-supplemented incubations are indicated by: * $P < 0.01$.

[b] PC: phosphatidyl choline, PE: phosphatidyl ethanolamine, SM: sphingomyelin.

removal of both the organic and inorganic constituents of bone but this is apparently done in step-wise fashion. *In vitro*, the organic matrix appears to be resistant to digestion until it has been at least partially demineralized (15). This suggests a temporal sequence of enzyme release, if indeed enzymes are associated with early calcium mobilization. It has been postulated that the early calcium mobilization is due to the production of lactic (16, 17) and citric acid (16–18) which is capable of dissolving the mineral (15). The secondary removal of matrix would then proceed by the action of a combination of collagenase (19, 20) and lysosomal enzymes (21, 22). Most experiments have demonstrated that these enzymes appear later than 24 hr following the administration of parathyroid hormone. That the delay is not due to a time lag required for enzyme activation after release is suggested by the rapid action of collagenase derived from bone cells by Puzas and Brand (23). To date, no report has appeared in the literature documenting the effect of parathyroid hormone on the lipolytic activity of bone. However, PTH has been shown to induce lipolysis in rat epididymal fat tissue *in vitro* (24).

The close correlation of the appearance of enzymes capable of degrading phosphatidyl choline with calcium release is, of course, only circumstantial evidence that the two events are related. The later appearance of collagenolytic activity parallels other reports in the literature and seems to indicate that the experimental conditions are, at least, analogous to what has been observed *in vivo*. Previous reports have indicated that phosphatidyl choline, phosphatidyl ethanolamine, and sphingomyelin are the principal phospholipids extractable from bone matrix before demineralization (7). Following demineralization, phosphatidyl serine becomes extractable. While phospholipids have been implicated in bone formation at the epiphyseal line (7), there has been no role assigned to them in the diaphysis and indeed some data would actually suggest that they are not actively involved in bone formation in that area (25). Johnson (26) described histochemically a lipid layer on bone surface which is inactive, i.e., being neither formed nor resorbed. It appears that this layer is made up of cellular extensions, is permeable to large molecules, and probably plays an important role in calcium homeostasis (27). In conditions such as the hypophysectomized state (28) and following estrogen administration (29) there is an accumulation of lipids which might well be associated with an increase in the number of inactive surfaces. If this lipid layer is real, and all information would suggest that it is, it is reasonable to assume that removal or alteration of the lipid by appropriate enzymes would be a necessary step to calcium mobilization and subsequent bone resorption. This, then, is one possible explanation for the sequence of changes which has been described. Alternatively, the lipid removal could be associated with an increased turnover of some components of the surface cell membrane. It now seems probable that osseous tissue is separated from extracellular fluid by these cell membranes (30) and that the membranes have the ability to control ingress and egress of ions. It is not known whether there is a change in the cell membrane during the process but it is conceivable that the early mobilization of calcium which occurs after the administration of parathyroid hormone is associated with enzymatic alteration of the membrane.

Summary. The effect of 2 units of PTH on 10-day-old rat tibiae was studied. Incubations were carried out at timed periods up to 48 hr. The following parameters were measured: (a) the release of ^{45}Ca from prelabeled bone; (b) the release of [^{3}H]proline and [^{3}H]hydroxyproline from prelabeled collagen; (c) the re-

lease of [*methyl*-^{14}C]choline from prelabeled phosphatidyl choline. There was an early release of ^{45}Ca from bone fragments that paralleled the degradation of phosphatidyl choline. Collagenolytic activity was not demonstrable until later and reached its maximum 24 to 48 hr after the start of incubation. The data indicated that there is an increase in the breakdown of phospholipids which occurs at the time calcium mobilization is taking place. Degradation of at least a portion of the organic matrix of bone occurs later.

1. Ellworth, R., and Futcher, P. H., Bull. Johns Hospital **47,** 91 (1935).
2. Seyle, H., Arch. Pathol. **54,** 625 (1942).
3. Brewer, H. B., N. Engl. J. Med. **282,** 909 (1970).
4. Raisz, L. G., J. Clin. Invest. **44,** 103 (1965).
5. Raisz, L. G., N. Engl. J. Med. **282,** (1970).
6. Cruess, R. L., and Clark, I., Biochem. J. **96,** 262 (1965).
7. Wuthier, R. E., J. Lipid Res. **9,** 68 (1968).
8. Irving, J. T., and Wuthier, R. E., Clin. Orthop. Rel. Res. **56,** 237 (1968).
9. Wuthier, R. E., and Eanes, E. D., Calc. Tissue Res. **19,** 197 (1975).
10. Folch, J., Lees, M., and Stanley, G. H., J. Biol. Chem. **226,** 497 (1957).
11. Marinetti, G. S., J. Lipid Res. **3,** 1 (1962).
12. Skipski, V. P., Peterson, R. F., and Barcley, M., Biochem. J. **90,** 374 (1964).
13. Wagner, H., Horhammer, L., and Wolff, P., Biochem. J. **334,** 75 (1961).
14. Kaufman, E. J., Glimcher, M. J., Mechanic, G. L., and Goldhaber, P., Proc. Soc. Exp. Biol. Med. **120,** 632 (1965).
15. Neuman, W. F., Mulryan, B. J., and Martin, G. R., Clin. Orthop. **17,** 124 (1960).
16. Nisbet, J. A., Hellwell, S., and Nordin, B. E. C., Clin. Orthop. **70,** 220 (1970).
17. Peck, W. A., and Kikrksen, T. R., Clin. Orthop. **48,** 243 (1966).
18. Wolinsky, I., and Cohn, D. V., Endocrinology **84,** 28 (1969).
19. Fullmer, J. M., and Lazarus, G. S., J. Histochem. Cytochem. **17,** 793 (1969).
20. Wood, J. F., and Nichols, G., J. Cell. Biol. **26,** 747 (1965).
21. Tolnai, S., Canad. J. Physiol. Pharmacol. **461,** 261 (1968).
22. Vaes, G., J. Cell. Biol. **39,** 676 (1968).
23. Puzas, J., and Brand, J., Biochim. Biophys. Acta **429,** 964 (1976).
24. Werner, S., and Low, J., Horm. Metabol. Res. **5,** 292 (1973).
25. Cruess, R. L., and Hong, K. C., Calc. Tissue Res. **13,** 305 (1973).
26. Johnson, L. C., *in* "Bone Biodynamics. Henry Ford Hospital International Symposium" (H. Frost, ed.), p. 543. Little, Brown, Boston, Mass. (1964).
27. Talmage, R. V., Cooper, C. W., and Park, H. Z., Vitamins Horm. **103,** 140 (1970).
28. Sakai, T., Yoshinari, T., and Cruess, R. L., Endocrinology **83,** 51 (1968).
29. Sakai, T., Cruess, R. L., Yoshinari, Y., and Iida, K., Endocrinology **86,** 167 (1970).
30. Neuman, W. F., Fed. Proc. **28,** 1846 (1960).

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