2. Kapral, F. A., Shayegani, M. G., J. Exp. Med., 1959, v110, 123.
3. Bernheimer, A. W., Schwartz, L. L., J. Gen. Microbiol., 1963, v30, 455.

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Effect of Short Term Alpha Irradiation on Parathyroid Activity and Osteoclast Numbers. (30103)

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It is now generally accepted that the end result of the action of the classical hormone of the parathyroids is to aid in the maintenance of constant ionic calcium levels of extracellular fluids. This condition is achieved primarily through the action of the hormone on both bone and kidney. A morphological indication of increased hormonal activity, particularly in the metaphyseal region of bone, is an increase in numbers of osteoclasts. This relationship between parathyroid function and osteoclast proliferation has been recognized for many years(1-3). Also, in earlier reports, the increase in number of these cells has been studied under standardized conditions, so that the osteoclast numbers in the metaphysis of the femur of the rat could be used as an index of endogenous parathyroid activity(4,15). However, the role of the osteoclast is still not completely understood, although there is evidence to support the suggestion that it is capable of actively reabsorbing bone(5-7). Whether the cell initiates the reabsorption or phagocytizes the remnants after bone resorption has been induced by other agents, has still not been clarified. This phagocytic action of the osteoclast has been utilized by Arnold and Jee to study incorporation of plutonium into these cells(8).

The purpose of the study reported here was to attempt to destroy osteoclast function through the radiation emitted by plutonium incorporated by phagocytosis. Under such conditions it was hoped to determine whether parathyroid hormone acted through osteoclasts in its control of calcium homeostasis. In previous studies(10,11) it had been shown that the technique of peritoneal lavage in the rat stimulated endogenous parathyroid activity which caused a constant but elevated calcium and hydroxyproline transfer from bone to extracellular fluid and a proliferation in osteoclasts. Therefore, at specific times after administration of the plutonium, the lavage technique was employed to increase endogenous hormone production so that the effect of plutonium on osteoclast numbers and on bone breakdown could be studied.

Materials and methods. All experiments were conducted at the Argonne National Laboratories. The plutonium was furnished in the form of $Pu(NO_3)_4$ in 1% sodium citrate at pH 6.5. Experimental animals were male Sprague-Dawley rats, 180-200 g, maintained on a calcium-free diet for 3 days prior to use. One microcurie of plutonium was injected intraperitoneally at *one* of the following times: (1) 5 days before peritoneal lavage, (2) 24 hours before experimental use, and (3) injections of one microcurie each, at 48 hours and 24 hours before use.

The experimental procedure consisted of 8 or 12 hours of continuous peritoneal lavage, using a calcium-free, phosphate-free buffered rinse. This method has been explained in detail(12). Calcium in the sera and in the hourly lavage samples was analyzed using a flame photometric method(13). The analysis for free hydroxyproline in the sera and lavage washes has been previously described(14).

Histology. Immediately after the lavage procedure, the rats were sacrificed and both femora and in some cases, mandibles, vertebrae and liver tissues were removed and fixed

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in a modified Bouin's solution. The femora were decalcified in 5% formic acid or in 18% EDTA at pH 7.0 for 3 days. All tissues were embedded in paraffin and 5 μ thick sections were prepared. Radioautographs were made by staining the sections with periodic acid and Schiff's reagent and then coating the slides with NTB2 Kodak emulsion. After development of the autographs, the tissue was counterstained with hematoxylin.

Sections of bone tissue adjacent to that used for autographs were stained, and osteoclasts were quantified according to the method of Toft and Talmage(15).

It was also possible to prepare radioautographs of tritium labelled compounds incorporated in the same tissue containing plutonium because it was found that EDTA decalcification removed all plutonium. These autographs were made from sections of femur after administration of 50 microcuries of H^3-proline to the plutonium treated rats.

Results. Distribution of plutonium. In the metaphysis, the only significant cellular concentration of plutonium was within the osteoclast (Fig. 1). In the 5-day injected group nearly every osteoclast contained alpha tracks. In the 24-hour and double injected groups approximately 50% of the osteoclasts contained plutonium. All groups also showed diffuse distribution of alpha tracks within the new osteoid of the trabeculae, whereas, the cartilage contained very little plutonium. While a few of the marrow cells contained radioactivity, none of the large megakaryocytes showed plutonium incorporation. Because of the close proximity of *osteoblasts* to the newly formed matrix which contained the plutonium, it was not possible to determine whether these cells actually incorporated the label, as the alpha tracks covering osteoblasts could have originated from the adjoining matrix.

In the diaphysis, the distribution of alpha tracks was relatively diffuse in all exposed mineralized surfaces (Fig. 2). No osteocytes appeared to concentrate the plutonium; especially when compared to the concentration found in the osteoclasts.

Effect on calcium and free hydroxyproline transfer. From the data in Table I, it is obvious that under the conditions of these experiments, the administration of plutonium produced no significant effect on calcium or free hydroxyproline levels of the wash removed during peritoneal lavage.

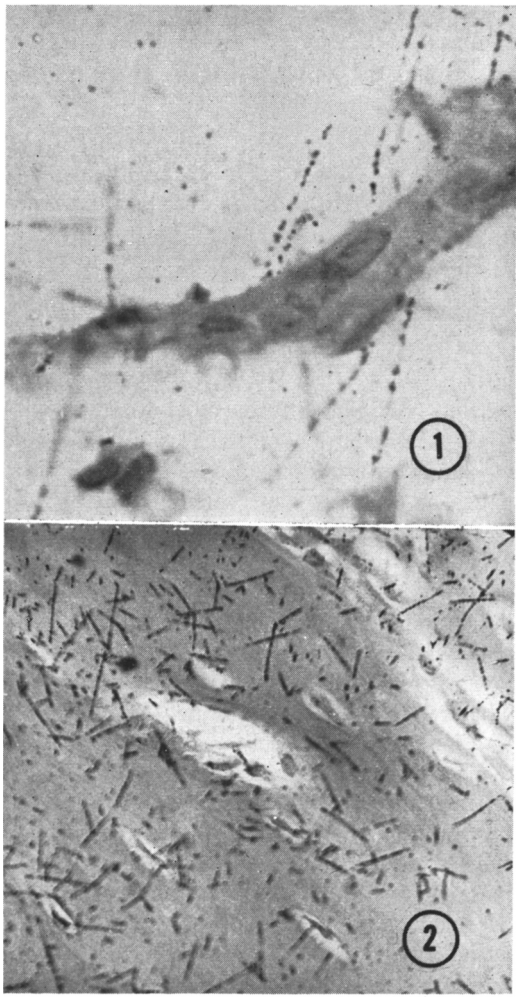

FIG. 1. Osteoclast from femur of a rat killed 24 hr after having received one microcurie of plutonium-239.

FIG. 2. General distribution of plutonium-239 in compact bone of diaphysis. The isotope is not concentrated within osteocytes.

Effect on osteoclast proliferation. The peritoneal lavage technique, using a calcium-free wash, as is routinely employed in our laboratories, produces a marked increase of osteoclast numbers in the metaphysis of the rat femur. As illustrated in Table II, the increase averages approximately 75% from a figure of 33 cells/2.6 sq mm to approximately

TABLE I. Effect of Plutonium-239 on Calcium and Free Hydroxyproline Levels.

Group	No. of animals	Mg % lavage calcium	Mg % serum calcium	μM/ml lavage fluid hydroxyproline
(A) Lavaged				
(1) 5 day*	5	5.8 ± .1		.025 ± .001
(2) 24 hr*	12	5.5 ± .1	9.3 ± .2	.022 ± .001
(3) Double injected	5	5.5 ± .1		
(4) Non-injected	15	5.7 ± .1	9.6 ± .3	.023 ± .001
(B) Non-lavaged				
(1) Non-injected	5		9.6 ± .3	

* Amount of time plutonium-239 was in animal.

57 cells after 8 hours of lavage. In the experimental animals, this average increase was also noted when the plutonium was administered 5 days before subjection to peritoneal lavage, and at this time most osteoclasts showed plutonium incorporation (Table II).

When plutonium was administered only 24 hours prior to the lavage, the lavage procedure failed to produce the expected stimulation in osteoclast numbers, although the radioisotope did not lower the normal population in the non-lavaged rat. In the third situation, plutonium was administered both at 48 hours and 24 hours before lavage, and this resulted in an even greater inhibition of osteoclastic proliferation (Table II). However, there appeared also to be a decrease in the osteoclast population of the non-lavaged, doubly injected rat.

Effect on incorporation of certain tritiated compounds. In the latter experiments, either H^3-thymidine or H^3-proline was administered to rats previously given plutonium. The thymidine was injected one hour prior to sacrifice at which time it is known to be incorporated only into the mesenchyme cell (undifferentiated cell) population of bone (19). The results of the thymidine administration are summarized in Table III. About 20% of the mesenchymal cells incorporated the H^3-thymidine label in the non-irradiated rats. This agrees with data reported by others(19,20). Plutonium administration (double injected) significantly reduced this incorporation to 12%.

TABLE II. Effect of Plutonium-239 on Osteoclast Number within the Femoral Metaphysis.

Group	No. of animals	Avg osteoclasts/ field (2.6 mm²)
(A) Lavaged		
I. 8 hr lavage		
(a) 5 day*	5	57.4 ± 3.1
(b) 24 hr*	12	41.9 ± 1.6†
(c) Non-injected	14	54.1 ± 2.6
II. 12 hr lavage		
(a) Double injected	5	35.4 ± 1.9
(B) Non-lavaged		
(a) 5 day*	5	35.9 ± .6
(b) 24 hr*	7	35.4 ± 2.3
(c) Non-injected	11	33.0 ± 1.5
(d) Double injected	12	27.7 ± 1.0‡

* Amount of time plutonium was in animal.
† Significantly lower than 5 day injected or non-injected ($P < .001$).
‡ Significantly lower than double injected, 12 hr lavage group ($P < .005$).

A preliminary experiment using only 6 animals is also included, in which H^3-proline was used to give some indication of possible effects of the plutonium on osteoblast function. No effect on proline uptake was apparent (Table III).

Discussion. In discussing the results reported above, emphasis will be placed on the problem of plutonium incorporation in bone, and how this influenced the formation of osteoclasts. This will be followed by a discussion regarding the question of parathyroid control of bone remodelling and/or maintenance of extracellular calcium levels.

The physiological effects of plutonium as a heavy metal are unknown. Its immediate incorporation into bone is considered to be related to the inorganic components to which it is bound(9). Once incorporated, it is not readily removed and eventually will cause severe radiation bone damage(16). In this study, however, the longest burden time was 5 days and no morphological bone damage could be demonstrated. The data presented here demonstrate that despite this lack of gross morphological change, the presence of

TABLE III. Effect of Plutonium-239 on Thymidine-H^3 and Proline-H^3 Uptake.

Group	No. of animals	% labelled osteoblasts	% labelled mesenchyme cells
(A) Thymidine			
(1) Pu^{239} double injected	3		12.0 ± .5*
(2) No Pu^{239}	3		19.2 ± .6
(B) Proline			
(1) Pu^{239} double injected	2	96.0 ± 1.0	
(2) No Pu^{239}	4	94.2 ± 1.2	

Notes: Animals sacrificed 30 min after tritiated proline injections; one hour after tritiated thymidine injection.
* Significantly lower than no Pu^{239} injected group ($P < .001$).

the heavy metal within 24 hours interfered with mitotic activity of the mesenchyme population and with the formation of osteoclasts. Five days later these effects are no longer seen, indicating a change in the distribution of plutonium during the intervening time. This transient and localized effect on mitotic activity and osteoclast formation is considered to support our recent postulate(22) which suggested that under increased parathyroid hormone activity, additional osteoclasts are derived directly from the undifferentiated mesenchyme population. The decreased thymidine uptake by these cells in the 24 hour plutonium treated rat and their subsequent failure to form additional osteoclasts under parathyroid stimulation suggest a basic interference by the isotope in the process of differentiation of these mesenchyme cells(17,18). However, whether the function of the mature differentiated bone cell is unchanged after short term alpha irradiation has yet to be determined. Our data, while limited, indicate that the osteoblast continues to take up tritiated proline, and that the process of collagen formation is normal. Since osteoclastic activity in the metaphysis is concerned with remodelling and breakdown of both old and newly formed bone, it is not surprising that by 5 days, most, if not all, of these cells contain plutonium, confirming the earlier observation of Arnold and Jee(8). The ability of osteoclasts to concentrate the plutonium suggests that the existing members of this population continue to function within this time period.

Our final point centers around the data showing that this type of experiment, though affecting osteoclast formation, does *not* prevent the expected parathyroid effect on calcium and hydroxyproline removal by peritoneal lavage. The ability of bone to respond to parathyroid stimulus and maintain the expected rate of calcium removal despite the plutonium effect on metaphyseal osteoclasts confirms similar findings produced by other agents, such as paraaminosalicylic acid, or after long term thyroidectomy(21,22). It appears, therefore, that the size of the viable population of osteoclasts in the metaphysis has little, if any, bearing on parathyroid control of calcium homeostasis. While these studies give no evidence of extensive damage to osteoclast function by 5 days, Arnold and Jee(8) reported that by 9 days after plutonium administration, most osteoclasts appeared to be damaged. As the result of extended plutonium treatment, these authors suggested that the bone remodelling process had been destroyed. Because of the heavy incorporation of plutonium into osteoclasts by 5 days, and subsequent damage to cells by 9 days(8), the function of metaphyseal osteoclasts in calcium homeostasis is open to question.

In conclusion, therefore, these and supporting data suggest that while the classical hormone of the parathyroids normally stimulates the formation of osteoclasts in those areas of bone where active remodelling occurs, this stimulation for osteoclastic proliferation is distinct and separate from the activity of this hormone in the maintenance of calcium homeostasis. These data do not permit us to suggest the area and cell types in bone responsible for rapid calcium mobilization, except to point out that they are not cells readily susceptible to damage by plu-

tonium. Nor can the effect be through osteoblasts, which are readily damaged by other agents without concomitant effects on calcium removal(22).

Summary. A study of the distribution of plutonium-239 in bone, and its effects on bone cells has been presented and correlated with endogenous parathyroid activity. There was no measurable effect on the function of existing osteoblasts or osteoclasts during this short term (5 day) experiment. Also, incorporation of plutonium into osteoclasts did not disrupt the ability of parathyroid hormone to maintain normal calcium levels. However, it could be demonstrated that plutonium, for the first 48 hours after administration, affected certain of the undifferentiated bone cells, which in turn prevented the increased osteoclast production normally seen following endogenous parathyroid stimulation.

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1. Pugsley, L., Selye, H., J. Physiol., 1933, v79, 113.

2. Gaillard, P. J., Exp. Cell Research, Suppl., 1955, v3, 154.

3. Walker, D. G., Bull. Johns Hopkins Hosp., 1961, v188, 80.

4. Talmage, R. V., Toft, R. J., In The Parathyroids (R. Greep, R. V. Talmage, eds.), Charles C Thomas, Springfield, Ill., 1961, pp224-40.

5. Jordan, H. E., Anat. Rec., 1920, v18, 268.

6. Kroon, D. B., Acta Anat., 1954, v21, 1.

7. Hancox, N. M., J. Biophys. Biochem. Cytol., 1961, v11, 651.

8. Arnold, J. S., Jee, W. S. S., Am. J. Anat., 1957, v101, 367.

9. Jee, W. S. S., Arnold, J. S., Health Physics, 1962, v8, 709.

10. Talmage, R. V., Doty, S. B., Gen. Comp. Endocrinol., 1962, v2, 473.

11. Bates, W. K., Awapara, J., Talmage, R. V., Proc. Soc. Exp. Biol. and Med., 1964, v115, 650.

12. Talmage, R. V., Elliott, J. R., Endocrinology, 1958, v62, 717.

13. Yates, C., Doty, S., Talmage, R. V., Proc. Soc. Exp. Biol. and Med., 1964, v115, 1103.

14. Bates, W. K., McGowen, J., Talmage, R. V., Endocrinology, 1962, v71, 189.

15. Toft, R. J., Talmage, R. V., Proc. Soc. Exp. Biol. and Med., 1960, v103, 611.

16. Jee, W. S. S., Arnold, J. S., Cochran, T. H., Twente, J. A., Mical, R. S., in Some Aspects of Internal Radiation, (T. F. Dougherty, ed.) Pergamon Press, New York, 1962.

17. Twente, J. A., Jee, W. S. S., Health Physics, 1961, v5, 142.

18. Kember, N. F., in Some Aspects of Internal Irradiation, (T. F. Dougherty, ed.) Pergamon Press, New York, 1962.

19. Young, R. W., J. Cell. Biol., 1962, v14, 357.

20. Tonna, E. A., Anat. Rec. 1960, v137, 251.

21. Doty, Stephen B., Talmage, R. V., Gen. Comp. Endocrinol., 1964, v4, 545.

22. Talmage, R. V., Doty, S., Cooper, C., Yates, C., Neuenschwander, J., in Parathyroid Gland; Ultrastructure secretions, and Functions, Univ of Chicago Press, 1964, in press.

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Some Endogenous Parathyroid Hormone Effects Manifested by Bone *in vitro*.* (30104)

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The incubation and organ culture of bone fragments have been used by several workers to examine the behavior of bone (*in vitro*) under the influence of parathyroid extract or exogenous parathyroid hormone (1-6). However, little work has been done to assess possible endogenous parathyroid effects in such systems(5).

Recent studies from our laboratory have dealt primarily with the effects of endogenous parathyroid hormone on bone metabolism *in vivo*(8). Some studies on incubated bone tissue have also been reported(7,9,10). The purpose of the studies presented here was

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