The effects of acetate, succinate and probenecid on the displacement of intracellularly accumulated PAH were studied at various pH values ranging from 7.4 to 8.9. Acetate, which inhibits the loss of accumulated PAH at pH 7.4(4), has no such noticeable effect at lower hydrogen ion concentrations. Succinate and probenecid stimulate the displacement of PAH at all tested hydrogen ion concentrations without exhibiting a noteworthy pH dependence.

Summary. Studies indicate that PAH accumulation by thin slices of rabbit renal cortex shows a sharp pH optimum at 8.1 to 8.3. At the pH optimum, acetate does not show stimulation of PAH uptake that is characteristic of its effect at physiological pH. The displacement of previously accumulated PAH does not show a sharp pH dependence.

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Inhibition by Actinomycin D of Bone Resorption Induced by Parathyroid Hormone or Vitamin A.* (30253)

LAWRENCE G. RAISZ† (Introduced by Harold C. Hodge)
Departments of Pharmacology and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Both parathyroid hormone (PTH) and vitamin A (vit. A) stimulate the slow development of bone resorption in tissue culture (1). PTH inhibits osteoblasts and stimulates fibroblastic and osteoclastic proliferation as well as inducing calcium release and resorption of bone matrix. Cell proliferation is less prominent with vit. A. In several recent studies(2-5), prior administration of Actinomycin D *in vivo* was found to inhibit the calcium mobilizing response to exogenous PTH but not its effect on phosphate transport. Since Actinomycin D inhibits DNA-dependent RNA synthesis, it was suggested that the calcemic effect of PTH depended upon new RNA synthesis. On the basis of similar experiments an RNA-dependent step has been postulated for a number of other hormones (6). The effect of Actinomycin D on the stimulation of bone resportion in tissue culture by both PTH and vit. A was examined in the present study, since these agents are thought to act by different mechanisms.

Methods. The methods employed have been described(1). Paired bone shafts of radius and ulna from 19-day rat embryos were cultured for 72 hours in 50% human serum in Eagle's medium in an atmosphere of 5% CO_2 in air. Embryonic bone rudiments were obtained from pregnant rats in which Ca^{45} was injected 36 hours prior to sacrifice. The ratio of the release of previously incorporated Ca^{45} from treated bones to that from paired controls served as a measure of bone resorption. In addition, each bone was examined histologically. Three groups of experiments were performed.

Group I. Bones were treated with submaximal doses of purified PTH (0.12 μg/ml of a purified preparation with a potency of 2500 U/mg, kindly provided by Dr. G. Aurbach) or with vit. A (3 μg/ml). Varying doses of Actinomycin D (kindly provided by Dr. N. G. Brink of Merck Sharp & Dohme Research Laboratories) were added to both the treated and paired control cultures. All agents were added to the medium prior to explantation of the bone rudiments. *Group*

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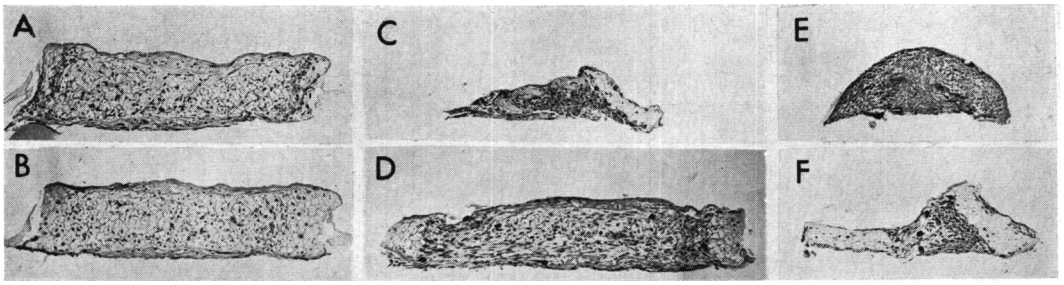

FIG. 1. Paired bone rudiments cultured for 72 hr. Hematoxylin and Eosin or Alizarin Stain (× 50). A and B (radii)—Control *vs* Actinomycin D 0.004 μg/ml; C and D (ulnae)—PTH 0.3 μg/ml *vs* PTH 0.3 μg/ml and Actinomycin D 0.004 μg/ml; E and F (radii)—PTH 3.0 μg/ml *vs* PTH 3.0 μg/ml and Actinomycin D 0.004 μg/ml.

II. Bones were cultured in the presence of varying doses of Actinomycin D for 4 hours prior to addition of PTH (0.12 μg/ml). In these experiments the response was followed by sampling the medium at different intervals of culture. In one experiment, bones were removed at 12 and 24 hours of culture as well as at 72 hours for histologic examination. In another experiment the bones were transferred, after 8 hours of PTH and 12 hours of Actinomycin D treatment, to a new medium which contained PTH alone. *Group III.* The effect of a single dose of Actinomycin D on the response to varying doses of PTH or vit. A was assessed. All agents were added simultaneously as in Group I. A less potent preparation of PTH, 2000 U/mg, was used. Ca^{45} release was measured at 24 and 72 hours and histological changes examined at 72 hours. In these experiments, one of the paired bones received Actinomycin D while both bones were treated with PTH or vit. A. Thus, treated/control ratios indicate Actinomycin D effects rather than PTH or vit. A effects.

Results. Cultures of embryonic bone shafts were extremely sensitive to Actinomycin D. The addition of 0.04 μg/ml (3×10^{-8} M) resulted in extensive cell death so that only a few nuclei remained at 72 hours, mainly in the muscle tissue adherent to the bone. With 0.004 μg/ml, Actinomycin D treated bones were less cellular than controls (Fig. 1A) although living cells remained. Despite these histologic changes, Ca^{45} release was little affected by Actinomycin D presumably because the release was due largely to exchange of calcium rather than to active resorption.

In the experiments in Group I, toxic doses of Actinomycin D inhibited both PTH and vit. A induced bone resorption when one of these agents and the inhibitor were present simultaneously (Fig. 2). A dose of 0.004 μg/ml of Actinomycin D significantly decreased Ca^{45} release in response to vit. A, whereas the response to PTH was not significantly decreased although it appeared to be less marked histologically. 0.0004 μg/ml of Actinomycin D did not inhibit either vit. A or PTH responses.

Even the largest doses of Actinomycin D did not inhibit the response to PTH completely, suggesting that some bone resorption may have taken place before the toxic inhibition by Actinomycin D. To test this, bones were pretreated for 4 hours with Actinomycin D before addition of PTH (Fig. 2). The previously ineffective dose of 0.004 μg/ml now

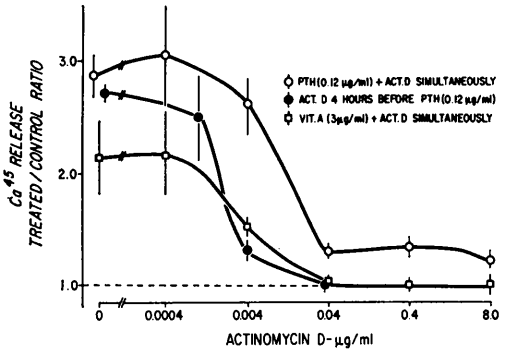

FIG. 2. Effect of varying doses of Actinomycin D on response to PTH or vit. A. Symbols are means ± standard error of 4 to 12 bone pairs for the ratio of Ca^{45} release from bones treated with vit. A or PTH *vs* paired controls. Both treated and control bones were cultured in medium containing the indicated doses of Actinomycin D.

caused significant inhibition of the PTH response. With a larger dose of Actinomycin D, all response to PTH was obliterated. When bones were treated with Actinomycin D alone for 4 hours and Actinomycin D plus PTH for 8 hours and subsequently cultured in PTH alone, there was a rapid return of PTH effect so that 16 hours after Actinomycin D was removed, the Ca^{45} release was actually greater than for bones which had been treated with PTH alone. Actinomycin D pretreatment at 0.001 μg/ml caused no significant inhibition of Ca^{45} release but did decrease cell proliferation in PTH-treated bones. Histological examinations at intervals showed that the cell necrosis with large doses of Actinomycin D was already extensive by 12 hours. Cell proliferation in response to PTH was not clearly evident until 24 hours.

In the experiments of Group III, the inhibition of PTH response by 0.004 μg/ml of Actinomycin D depended upon the dose of PTH employed (Fig. 3). Actinomycin D inhibited the response to a submaximal dose of parathyroid hormone but not to a high dose. Assuming the molecular weight of PTH to be 10,000, then the submaximal dose of 0.3 μg/ml represents 3×10^{-8} M. The effect of this dose was inhibited by adding 3×10^{-9} M Actinomycin D, but when the molar ratio of PTH/Actinomycin D was raised from 10 to 100, this inhibition was overcome. The inhibition of cell proliferation induced by Actinomycin D could also be overcome by adding PTH (Fig. 1). There was some stimulation of cell proliferation with 0.03 μg/ml of PTH (3×10^{-9} M) although no significant Ca^{45} release occurred. Actinomycin D added to this dose of PTH inhibited cell proliferation as much as it did in controls. Both the resorption and the dense fibroblastic proliferation in response to 0.3 μg/ml of PTH were inhibited by Actinomycin D, but compared with bones treated with Actinomycin D alone or Actinomycin D and 0.03 μg/ml of PTH, there was more fibroblastic proliferation and more Ca^{45} release. Three μg/ml of PTH produced almost complete resorption leaving a remnant of fibrous tissue. With this dose plus Actinomycin D, the histologic appearance and the Ca^{45} release resembled that in response to 0.3 μg/ml of PTH without Actinomycin D.

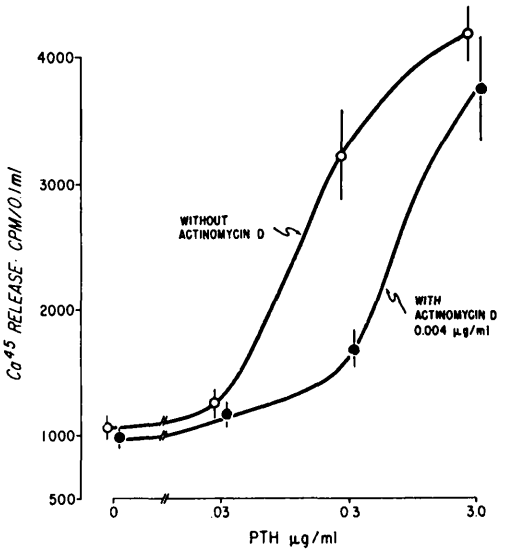

FIG. 3. Effect of Actinomycin D on response to varying doses of PTH. Symbols are means ± 1 standard error for Ca^{45} release from 3 to 4 individual bones from the same litter.

When the responses to vit. A and PTH were compared directly (Table I) it was apparent that PTH was a more potent stimulus for bone resorption. Vit. A also caused less cell proliferation than PTH. The effect of vit. A, even in maximal doses, was inhibited by Actinomycin D. Bones treated with high doses of vit. A alone showed considerable cell death and there may have been additive toxic effects of Actinomycin D and vit. A.

TABLE I. Effect of Actinomycin D on Response to Varying Doses of PTH or Vit. A.

	Ca^{45} release cpm/0.1 ml		
	Without Actinomycin D	With Actinomycin D (.004 μg/ml)	% inhibition
Control	3830 ± 170	3650 ± 110	4 ± 4
PTH, 0.1 μg/ml	4740 ± 410	4370 ± 660	8 ± 12
PTH, 1.0 "	11000 ± 410	8280 ± 720	25 ± 6
Vit. A, 1 "	4570 ± 300	4130 ± 310	10 ± 3
3 "	5743 ± 190	4090 ± 252	29 ± 4
10 "	7050 ± 650	5470 ± 700	31 ± 5

Values for Ca^{45} release are mean ± S.E. of 3 to 4 bones.

Values for % inhibition are mean ± S.E. for 3 to 4 bone pairs, calculated as % decrease in Ca^{45} release when Actinomycin D is added to one of the cultures.

Discussion. The sensitivity of embryonic bone in tissue culture to Actinomycin D is not surprising since these explants contain growing and dividing cells. Mammalian cells in tissue culture are highly sensitive to Actinomycin D during the phase of rapid RNA and protein synthesis that precedes cell division(7). PTH induces proliferation of fibroblasts and osteoclasts in bone at the same time that resorption is stimulated. Borle and Neuman(8) have shown that PTH could also stimulate cell proliferation in cultures of HeLa cells. In the present study, very small doses of Actinomycin D could prevent or limit the proliferative cellular response to PTH. Gaillard(9) has recently described similar results. If resorption is dependent upon these cellular changes, then Actinomycin D could inhibit resorption by preventing the synthesis of the new RNA required for these cells.

Vit. A causes less resorption and less cell proliferation than PTH. It has been postulated that vit. A induces resorption by proteolysis of matrix due to release of enzymes from lysosomes(10). If preformed lysosomal enzymes were present in abundance in embryonic bone, vit. A effects might occur despite Actinomycin D treatment. The sensitivity of the vit. A response to Actinomycin D suggests that the synthesis of new enzymes directed by RNA is required.

While these findings indicate that new RNA synthesis is an essential step in bone resorption induced by PTH or vit. A, inhibition by Actinomycin D need not indicate that the primary action of either PTH or vit. A is on DNA-dependent RNA synthesis. However, the findings that Actinomycin D inhibition can be overcome by increasing the dose of PTH and that Actinomycin D is more effective when given before PTH suggest that these two agents might compete at the nuclear site of Actinomycin D action. Competition for transport into the cell or a chemical interaction between these agents in the medium might also explain the results.

Summary. Actinomycin D inhibits cell proliferation in control cultures of embryonic bone and prevents the stimulation of bone resorption by parathyroid hormone (PTH) or vit. A. With a dose of 0.004 μg/ml of Actinomycin D, PTH effects are only partially inhibited and this inhibition can be overcome by increasing the dose of PTH. These findings suggest that these agents act competitively.

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