Hypercalcemia induced with an arotinoid in thyroparathyroidectomized rats. New model to study bone resorption in vivo.

U Trechsel, … , A Stutzer, H Fleisch

J Clin Invest. 1987;80(6):1679-1686. https://doi.org/10.1172/JCI113257.

Research Article

A model of stimulated bone resorption was developed using a synthetic retinoid in thyroparathyroidectomized rats. The retinoid induced an increase in bone resorption and in the number of vertebral subperiosteal osteoclasts. The resulting increase in plasma Ca could be used as an easily measured index of bone resorption. Three bisphosphonates produced a dose-related prevention and reversal of retinoid-induced hypercalcemia. Their potencies were similar to those previously obtained by histomorphometry. Irradiation (600 rad) of the rats prevented hypercalcemia but failed to reverse it, showing that proliferation of osteoclast precursor cells was important in inducing, but not in maintaining, bone resorption. Calcitonin produced similar effects on calcemia and prevented the increase in osteoclast number but failed to reverse the increase, suggesting that it inhibited precursor proliferation. This model represents a new tool to study mechanisms of bone resorption and the action of inhibitors in vivo.

Hypercalcemia Induced with an Arotinoid in Thyroparathyroidectomized Rats

New Model to Study Bone Resorption In Vivo

U. Trechsel, A. Stutzer, and H. Fleisch
Department of Pathophysiology, University of Bern, Bern, Switzerland

Abstract

A model of stimulated bone resorption was developed using a synthetic retinoid in thyroparathyroidectomized rats. The retinoid induced an increase in bone resorption and in the number of vertebral subperiosteal osteoclasts. The resulting increase in plasma Ca could be used as an easily measured index of bone resorption. Three bisphosphonates produced a dose-related prevention and reversal of retinoid-induced hypercalcemia. Their potencies were similar to those previously obtained by histomorphometry. Irradiation (600 rad) of the rats prevented hypercalcemia but failed to reverse it, showing that proliferation of osteoclast precursor cells was important in inducing, but not in maintaining, bone resorption. Calcitonin produced similar effects on calcemia and prevented the increase in osteoclast number but failed to reverse the increase, suggesting that it inhibited precursor proliferation. This model represents a new tool to study mechanisms of bone resorption and the action of inhibitors in vivo.

Introduction

Bone resorption by osteoclasts is a process which is tightly controlled by a number of circulating and local factors (1–3). Regulation can occur at different levels, namely by altering the activity of osteoclasts present in bone or by influencing the generation of new osteoclasts from precursor cells. Osteoclasts are thought to originate from stem cells in the bone marrow under the influence of hemopoietic growth factors. Multinucleated osteoclasts are then generated by fusion of precursor cells (4, 5). The activation of osteoclasts can be studied using the acute hypercalcemic effect of PTH in parathyroidectomized rats. This system has been successfully used to study the inhibitory effect of bisphosphonates on bone resorption (6). In contrast, an animal model of bone resorption in which this process is altered mainly by a recruitment of new osteoclasts and which allows to monitor this process by an easily measured biochemical parameter such as plasma calcium has, to our knowledge, not been reported.

Because it has long been known that excess vitamin A leads to an increase of osteoclastic bone resorption and of the number of osteoclasts (7–9), we have studied whether a synthetic vitamin A derivative (retinoid) shown in Fig. 1 might be used to develop such a model. The arotinoid used is one of the most potent retinoids known in terms of vitamin A activity (10, 11).

In this paper we demonstrate that the retinoid is a potent stimulator of bone resorption in the thyroparathyroidectomized (TPTX)[1] rat and that the increase in bone resorption involves the generation of new osteoclasts. The changes in bone resorption can be easily monitored by measuring plasma Ca. This system proved to be suitable to study the effect of inhibitors of bone resorption and to differentiate between their effects on osteoclast activity and on proliferation of precursors.

Methods

Except for the balance and ^{45}Ca kinetics study, male Wistar rats weighing 150–180 g from our breeding colony were used. The animals were thyroparathyroidectomized surgically under ether anesthesia and from then on received 1 μg thyroxin subcutaneously three times a week. The success of parathyroidectomy was tested by measuring plasma Ca 8 d after the operation. Rats with < 2 mM plasma Ca were considered TPTX. The TPTX rats were placed in single cages and pair-fed a commercial chow containing 1 g Ca/100 g, 0.75 g P/100 g, and 800 IU vitamin D_3/kg (Kliba 331, Klingenthalmühle, Basel, Switzerland). Blood was taken either by retroorbital puncture, aortic puncture, or from the tip of the tail and collected in heparinized tubes. Plasma was then obtained by centrifugation.

The retinoid ethyl *p*-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtyl]benzoate (Ro 13-6298, a gift from Hoffmann La Roche AG, Basel, Switzerland) (Fig. 1), was administered subcutaneously in 0.1 ml/rat of polyethylene glycol 300 with 10% ethanol. Control animals received the vehicle alone.

The bisphosphonates dichloromethylenebisphosphonate (Cl_2-MBP), 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (AHPrBP) (both provided by Procter & Gamble Co., Cincinnati, OH), and 4-amino-1-hydroxybutylidene-1,1-bisphosphonate (AHBuBP) (from Gentili S.p.A., Pisa, Italy) were dissolved in water, the pH was adjusted to 7.4, and NaCl was added to obtain isotonic solutions. The drugs were given subcutaneously in 2 ml/kg body wt.

Bovine parathyroid hormone (TCA powder, Sigma Chemical Co., St. Louis, MO) was injected subcutaneously in 0.2 ml/rat of 0.15 NaCl containing 0.01 M HCl, 1 mg/ml bovine serum albumin, and 0.16 M glutathion.

Parts of this work were presented at annual meetings of the American Society for Bone and Mineral Research (1984 and 1985), the XVIII European Symposium on Calcified Tissues (1984), and the Sixth Workshop on Vitamin D (1985). This work has appeared in abstract form (1984. *Calcif. Tissue Int.* 36:473 and 36[Suppl.]:S49) (1984. *In* Vitamin, Chemical, Biochemical and Clinical Update. A. W. Norman, K. Schaefer, H.-G. Grigoleit, and D. V. Herrath, editors. Walter de Gruyter, Berlin. 51).

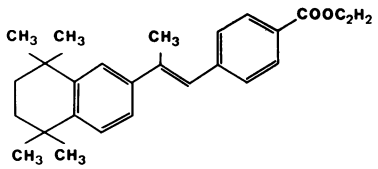
Address correspondence to Dr. U. Trechsel, Department of Pathophysiology, University of Berne, Murtenstr. 35, CH-3010 Berne, Switzerland.

Received for publication 6 February 1986 and in revised form 27 April 1987.

J. Clin. Invest.

0021-9738/87/12/1679/08 $2.00
Volume 80, December 1987, 1679–1686

1. *Abbreviations used in this paper:* AHBuBP, 4-amino-1-hydroxybutylidene-1,1-bisphosphonate; AHPrBP, 3-amino-1-hydroxypropylidene-1,1-bisphosphonate; Cl_2MBP, dichloromethylenebisphosphonate; CT, calcitonin; 25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D; TPTX, thyroparathyroidectomy; TRAP, tartrate-resistant acid phosphatase.



Ethyl p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-napthyl)-1-propenyl] benzoate

Figure 1. Chemical structure of the arotinoid Ro 13-6298.

Salmon calcitonin (CT) (Calcitonin-Sandoz, 100 Medical Research Center units/ml; Sandoz AG, Basel, Switzerland) was diluted in 0.1 M Na acetate with 1% bovine serum albumin and injected subcutaneously in a volume of 1 ml/kg body wt.

Balance and ^{45}Ca kinetics. This method was originally described by Aubert and Milhaud (12) and later modified as described earlier (13, 14). Weaning female Wistar rats from our own breeding colony were fed a commercial chow (Kliba 331) containing 1.0 g/100 mg Ca, 0.75% P, and 800 IU vitamin D_3/kg. At the age of 42 d, the animals were placed in single metabolic cages and pair-fed a diet containing 0.5% Ca and 0.35% P. This diet was made by adding Ca gluconate and a mixture of $K_2HPO_4/KH_2PO_4 \cdot 2H_2O$ (ratio 7:3) to a low-Ca, low-P, low-vitamin D diet (Altromin 1730, Altromin GmbH, Lage, FRG). The animals were supplemented with 50 IU of vitamin D_3 per d given orally in 0.1 ml vegetable oil. Thyroparathyroidectomy, thyroxin administration, and retinoid treatment were performed as described above. Daily treatment with the retinoid was started on the 54th day of life and given for 9 d.

Balance and ^{45}Ca kinetics were studied during the last 3 d of treatment. On day 60 of life, 30 μCi of $^{45}CaCl_2$ (sp act, 10–40 mCi/mg, Amersham International, Buckinghamshire, England) in 0.3 ml 0.15 M NaCl were injected into the tail vein of each rat and blood samples were taken at 2, 4, 6, 24, 48, and 72 h postinjection and analyzed for radioactivity. A balance study was performed over 72 h in which urine and feces were collected and ^{45}Ca radioactivity as well as Ca were determined in each. At the end of the experiment the plasma Ca level was measured.

The measurements of nonradioactive Ca allowed the determination of (*a*) intake of Ca, (*b*) net intestinal Ca absorption, and (*c*) Ca retention in the body. The latter is assumed to be entirely due to skeletal retention. The measurements of ^{45}Ca allowed, using a model with two compartments, the calculation of (*a*) bone formation defined as the amount of Ca which leaves the first compartment irreversibly over the time period measured and is not accounted for by Ca appearing in urine and the feces, (*b*) bone resorption calculated as the difference between the rates of bone formation and the Ca retention in the body, and (*c*) urinary Ca calculated by dividing the total urinary ^{45}Ca by the integral of plasma specific radioactivity. All fluxes are expressed as millimoles per day.

Determination of bone mineral content. Tibiae and spines were removed and freed from remaining soft tissue by Dermestid beetles (Dermestes maculatus) as described previously (15). The cleaned bones were dried at 105°C for 24 h and dry weight determined. Defatted dry weight was obtained by extraction with a mixture of equal parts of chloroform and methanol for 48 h, with one change of solution after 24 h. Subsequently the bones were dried at 105°C for 24 h and weighed. They were then ashed at 700°C for 3 d and the ash weight was determined. Finally, for Ca measurement, the ash was dissolved in 30 ml 1 M HCl.

Histology. Lumbal vertebrae were dissected without removing adherent muscles and periosteal tissue. The vertebrae were fixed in neutral formaldehyde, dehydrated in alcohol, cleared in xylene, and embedded in methylmethacrylate. Longitudinal sections of the central portion of the vertebrae were obtained using a Jung 1130 microtome. 5-μm sections were stained histochemically for tartrate-resistant acid phosphatase (TRAP) using naphtol-ASTR-phosphate as substrate and 6.5 mM disodiumtartrate. Active osteoclasts were identified as multinucleated cells showing strong TRAP activity by an intensely red cytoplasm. For microradiography, the tibia was embedded in methylmethacrylate. Frontal sections of the head of the tibia were then cut with a precision saw and ground to a thickness of 80 μm.

Irradiation experiments. TPTX rats were subjected to 600 rad x-ray irradiation using a conventional x-ray source. This radiation dose has been shown to suppress bone marrow proliferation in the rat (16).

Analytical methods. Ca was measured by atomic absorption spectrophotometry and inorganic phosphate by the method of Chen et al. (17). 25-Hydroxyvitamin D (25[OH]D) and 1,25-dihydroxyvitamin D ($1,25[OH]_2D$) were measured by protein binding assays as described previously (18).

Statistical analysis. Unless stated otherwise, all values are expressed as means±SEM. Significance of differences between groups was evaluated by the two-sided Student *t* test.

Results

Balance and ^{45}Ca-kinetics. Preliminary experiments had shown that the retinoid increased plasma Ca in TPTX rats. Although these results suggested that the compound might stimulate bone resorption, hypercalcemia could also have been due to the action of the retinoid on Ca metabolism at another site. A balance and ^{45}Ca-kinetics study was therefore performed to identify the sites of action of the compound on Ca metabolism. Because the retinoid effect appeared to require a few days to develop the Ca balance and ^{45}Ca kinetics measurements were started after 6 d of retinoid administration.

The main findings are presented in Fig. 2, *A* and *B*. Fig. 2 *A* demonstrates that the retinoid produced a conspicuous increase of Ca release from bone, i.e., bone resorption, whereas Ca entry into bone, i.e., bone formation, was unaffected. Plasma Ca was significantly increased at the highest dose level tested. Plasma phosphate was unchanged (data not shown). As shown in Fig. 2 *B*, retinoid treatment also produced a dose-related decrease of net intestinal Ca absorption. It should be noted that Ca intake was similar in control animals and in rats receiving 0.2 or 1 μg retinoid/d (1.25±0.04 mmol Ca/d, mean±SD). Retinoid given at 5 μg/d, however, led to a decrease of food intake, so that those animals only received 1.0±0.07 mmol Ca/d. Despite this, urinary Ca was increased

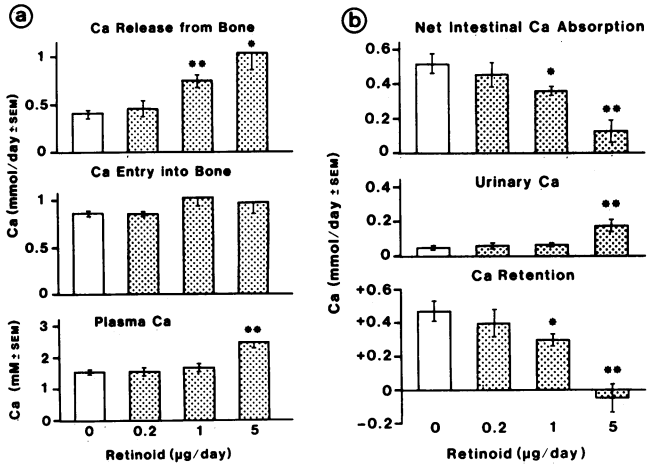


Figure 2. Effect of retinoid on Ca balance and ^{45}Ca kinetics in TPTX rats. Animals were treated with retinoid for 9 d, and Ca balance and ^{45}Ca kinetics were performed during the last 3 d of treatment, as described in Methods. Values are means±SEM of four animals (0 and 1 μg) or three animals (0.2 and 5 μg) per group. $^*P < 0.05$; $^{**}P < 0.01$, as compared with controls by Student's two-sided *t* test.

at the highest dose level tested. Ca retention, which is the difference between net intestinal Ca absorption and urinary Ca output, decreased in parallel with Ca absorption in the gut, because the effect of the retinoid on urinary Ca was small compared to that on the intestine.

These findings suggest that the retinoid affected Ca metabolism mainly at the level of stimulated bone resorption and of the intestine by decreasing Ca absorption. The observed increase in plasma Ca appeared therefore likely to be due to the increased Ca release from bone.

Effect of retinoid treatment on plasma 1,25(OH)$_2$D in TPTX rats. Because 1,25(OH)$_2$D is considered to be the main physiological stimulator of intestinal Ca absorption, we assessed whether a decrease in plasma 1,25(OH)$_2$D might explain the impairment of Ca absorption in retinoid-treated TPTX rats. As shown in Table I, plasma 1,25(OH)$_2$D was decreased to very low levels at the highest dose level tested. Concomitantly, there was a conspicuous increase in plasma Ca, whereas plasma inorganic phosphate was unchanged. Another experiment with intermediate doses of retinoid showed a high inverse correlation ($r = 0.75$; $P < 0.001$) between plasma Ca and 1,25(OH)$_2$D (Fig. 3), suggesting a link between these two parameters. 25(OH)D was 11.8±2.2 nmol/liter (mean±SD) and did not significantly differ between groups.

Therefore, it appeared that the retinoid-induced decrease in intestinal Ca absorption might well be explained by a decrease in plasma 1,25(OH)$_2$D. In contrast, the stimulation of bone resorption was unlikely to be related to a difference in vitamin D metabolism.

Retinoid-induced hypercalcemia in TPTX rats. In view of the likely possibility that the observed increase in plasma Ca in rats given the largest dose of retinoid was due to the increased Ca release from bone, we then investigated whether plasma Ca might be used as an easily measured biochemical parameter to monitor bone resorption in retinoid-treated TPTX rats. In a first step, the time course of the induction of hypercalcemia and the conditions to maintain it were studied. To produce a rapid increase in bone resorption, the maximal tolerated dosage was also determined.

A single injection of 100 μg of retinoid produced a progressive increase of plasma Ca that was detectable after 24 h and continued over 4 d. Signs of hypervitaminosis A appeared on the second day after retinoid administration. In subsequent experiments, the retinoid was given three times at 25 μg/rat on three consecutive days (Fig. 4, *A* and *B*). With this schedule, signs of general toxicity, namely a decreased food intake, conjunctivitis, and hair loss, did not appear until day 4, and the time course of the increase in plasma Ca was indistinguishable from that observed with a single injection of 100 μg/rat. Plasma Ca reached a maximum on day 4 and then decreased to control values within 2–3 d. The level could be maintained or elevated by continuing retinoid administration, given either daily at a lower dose or intermittently. Fig. 4 *B* shows that one additional injection of retinoid given on day 4 at the same dose was able to maintain plasma Ca until day 6.

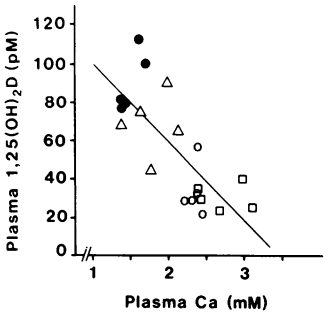


Figure 3. Relationship between plasma Ca and 1,25(OH)$_2$D in TPTX rats treated with retinoid. Animals were treated with solvent (●), 2.5 μg (△), 5 μg (○), or 7.5 μg (□) of retinoid per day for 6 d. Blood was obtained by aortic puncture 24 h after the last injection of retinoid, after an overnight fast.

Effect of retinoid treatment on bone mass in TPTX rats. To assess the extent of bone destruction under the conditions used in the experiments of Fig. 4, *A* and *B*, we used these treatment schedules to determine bone mass as assessed by measuring dry weight, defatted dry weight, ash weight, and Ca content in tibiae as well as vertebrae. As shown in Table II, no significant effect on bone mass could be detected 3 d after the onset of retinoid treatment. 6 d after beginning treatment, however, all parameters were lower than in the corresponding solvent-treated animals, statistical significance being reached in all, with the exception of Ca content of the tibia.

These results confirm that retinoid treatment stimulated bone resorption. Moreover, they show that plasma Ca is a more sensitive parameter to evaluate this effect than mineral analysis of the bones.

Effect of retinoid of osteoclast number in TPTX rats. To test whether the retinoid induced bone resorption by activating

Table I. Effect of Retinoid Treatment on Plasma Ca, Inorganic Phosphate, and 1,25(OH)$_2$D in TPTX Rats

Plasma concentration	Retinoid 0	0.1	1	10
	μg/d	*μg/d*	*μg/d*	*μg/d*
Ca (*mM*)	1.40±0.05	1.35±0.08	1.43±0.05	2.40±0.20*
P_i (*mM*)	1.77±0.06	1.84±0.06	2.03±0.10	1.74±0.10
1,25(OH)$_2$D (*pM*)	132±20	114±12	110±15	<26*

Values are means±SE, $n = 6$.
* $P < 0.005$ by Wilcoxon's rank test for unpaired values. TPTX rats were pair-fed and treated with one daily subcutaneous injection of retinoid for 6 d. They were then fasted overnight and blood taken by aortic puncture on the next day.

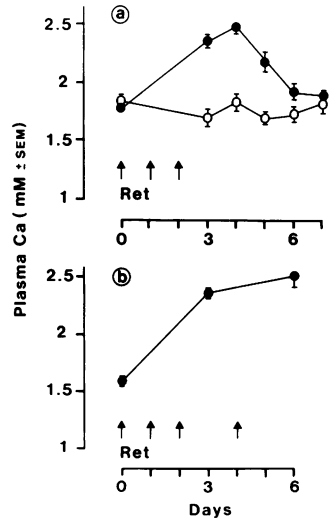


Figure 4. Effect of retinoid on plasma Ca in TPTX rats. Animals were given solvent (○) or 25 μg of retinoid (●) on days 0, 1, and 2 (*a*), or on days 0, 1, 2, and 4 (*b*). Values are means±SEM. No. of animals was 4 in the solvent group (*a*) and 5 in all other groups.

Table II. Effect of Retinoid on Bone Mass and Mineral Content in TPTX Rats

			Day 3		Day 6	
		Initial	Solvent	Retinoid	Solvent	Retinoid
No. animals		11	11	11	12	12
Dry weight (*mg*)	Tibia	516±9	535±7	523±9	557±6‡	509±7§
	Vertebra	491±8	515±7*	502±11	513±8	446±7‡,§
Defatted dry weight (*mg*)	Tibia	497±9	512±6	509±8	536±5‡	491±7§
	Vertebra	474±7	497±7*	487±10	518±8‡	447±7*,§
Ash weight (*mg*)	Tibia	276±5	289±4	292±6	310±3	291±5*,‖
	Vertebra	260±4	268±6	274±6	284±4‡	249±4§
Calcium (*mg*)	Tibia	111±2	119±2*	120±3‡	126±3‡	120±2¶
	Vertebra	104±2	107±2	111±3	115±4*	101±1‖
Plasma Ca (*mM*)		1.30±0.05	148±0.05	257±0.04§	1.39±0.04	2.93±0.08§

The animals were treated with 25 μg/rat of retinoid using the same injection schedule as in the experiments of Fig. 2. After an overnight fast, blood was taken by aortic puncture and the bones were removed and processed as described in Methods. Values are means ± SEM. *$P < 0.05$, ‡$P < 0.001$, ¶$P < 0.01$ as compared with initial values. §$P < 0.001$, ‖$P < 0.01$ as compared with animals treated with corresponding solvent.

osteoclasts present in bone and/or by stimulating the generation of new osteoclasts, we determined osteoclast numbers. As shown in Table III, 3 d of retinoid treatment resulted in a conspicuous increase in subperiosteal osteoclast number in vertebral bodies. An increase was also evident in the proximal tibial metaphysis, but in this region the osteoclasts were too numerous to be counted and compared accurately. It therefore appeared that retinoid produced an increase in the generation (recruitment) of new osteoclasts.

Effect of bisphosphonates on retinoid-induced hypercalcemia in TPTX rats. To investigate further whether plasma Ca reflects bone resorption in the retinoid model, we studied the effect of bisphosphonates on this parameter. Some of these compounds have been shown to be potent and specific inhibitors of bone resorption (19). The bisphosphonates used in this study have been shown previously to inhibit bone resorption in rats and to differ markedly in potency (20).

The drugs were given in two different situations. In the first we tested whether they could prevent the induction of hypercalcemia by the retinoid ("preventive" effect), and then whether they could reverse the hypercalcemia after it had been induced with retinoid ("curative" effect). The experimental protocols are shown in Fig. 5.

As shown in Fig. 6, all three bisphosphonates produced a dose-related inhibition of retinoid-induced hypercalcemia. The dose-response curves were almost identical in the preventive and the curative protocols. At the highest dose-levels tested, plasma Ca was in the range observed in untreated TPTX rats, indicating that the hypercalcemic effect of the retinoid was totally abolished. The dose-response curves were the same as those obtained previously in rats using histomorphometry (19). The radiomicrographs of Fig. 7 demonstrate that retinoid treatment resulted in a marked thinning of the cortical shaft of the head of the tibia, which, moreover, had an increased porosity (Fig. 7 *B*). These changes were completely inhibited by all three bisphosphonates given at a dose that prevented retinoid-induced hypercalcemia (Fig. 7, *C–E*).

Effect of irradiation on retinoid-induced hypercalcemia in TPTX rats. To assess whether the retinoid effect on recruitment involved the proliferation of precursor cells or only the formation of osteoclasts by fusion from postmitotic precursors, TPTX rats were irradiated with a dose known to abolish the proliferation of bone marrow cells (16), and plasma Ca was measured to monitor the effect of retinoid treatment on bone resorption.

As shown in the left panel of Fig. 8, irradiation of TPTX rats with 600 rad resulted in a complete abolition of retinoid-induced hypercalcemia, suggesting that proliferation of osteoclast precursors plays a central role in the induction of bone resorption by the retinoid. To ascertain that the irradiation did not act by inhibiting the osteoclasts already present, we assessed the hypercalcemic effect of PTH (6). TPTX rats were

Table III. Effect of Retinoid Treatment on Number of Osteoclasts in the Vertebral Periosteum of TPTX Rats

	Total no. of subperiosteal osteoclasts per vertebral section	No. of osteoclasts per millimeter of periosteal surface
		$\bar{x} + SEM$
Control	1–7	0.39±0.27
Retinoid	45–56	5.65±0.33*

Animals were treated with retinoid for 3 d, as in the experiment of Fig. 4*A*. Bones were removed 24 h after the last dose of retinoid and processed for histology as described in Methods. $n = 3$; *$P < 0.001$.

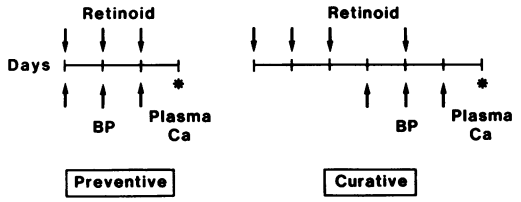


Figure 5. Protocol used to study the effect of bisphosphonates (*BP*) on retinoid-induced hypercalcemia. Dose of retinoid was 25 μg/rat per d.

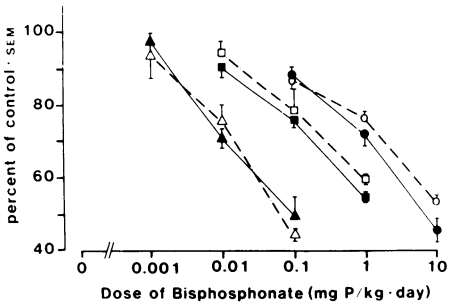


Figure 6. Effect of bisphosphonates on retinoid-induced hypercalcemia in TPTX rats. All animals were treated with retinoid and bisphosphonates according to the protocols shown in Fig. 5. To allow the comparison of results from three different experiments, values were normalized by taking plasma Ca in retinoid-treated control TPTX rats as 100%. Values are means±SEM, n = 4–6. Solid symbols are preventive; open symbols are curative. (- · · ○ · · -, — ● —) Cl_2MBP; (- · · □ · · -, — ■ —) AHPrBP; (- · · △ · · -, — ▲ —) AHBuBP.

irradiated and then immediately given 300 IU of PTH subcutaneously. Plasma Ca was measured before and 6 h after the administration of the hormone. In irradiated rats, the PTH-induced increase in plasma Ca was 0.47±0.08 mM ($\bar{x}$±SEM, n = 4). This value was not significantly different from the increase of 0.62±0.08 mM observed in control TPTX rats. Therefore, osteoclasts present remained responsive to PTH after irradiation.

Fig. 8, right panel, shows that irradiation, in opposition to bisphosphonates, had no effect on retinoid-induced hypercalcemia once it had been induced. This lack of effect also shows that osteoclasts once formed are not influenced by irradiation, and suggests that in the conditions used to study the curative effect of bone resorption inhibitors, proliferation of osteoclast precursors plays no significant role in maintaining bone resorption at a high level.

Effect of CT on retinoid-induced hypercalcemia in TPTX rats. CT is known to be a potent specific physiological inhibitor of osteoclastic bone resorption. Therefore, the effect of this hormone on retinoid-induced hypercalcemia was studied using the preventive and curative protocols (Fig. 5).

The effect of CT was assessed in two ways. First, plasma Ca was measured 24 h after the last injection of CT. This permitted to exclude the acute hypocalcemic effect of CT which lasts only a few hours. In addition, the acute hypocalcemic effect was also assessed by measuring plasma Ca 2 h after the injection of CT.

As shown in Fig. 9, CT given simultaneously with retinoid prevented the increase of plasma Ca as assessed 24 h after the last injections of CT and retinoid. The same dose of CT failed, however, to reverse retinoid-induced hypercalcemia once it had been induced. Similar results were obtained at a lower dose of 4 IU/kg in both protocols. These results are strikingly similar to those obtained with irradiation. Thus, it appears that CT prevented the recruitment of osteoclast precursors by retinoid.

In identical experiments, osteoclast numbers were then determined in groups sacrificed either at the end of the preventive (day 3) or the curative protocol (day 6). These results are shown in Table IV. In the preventive protocol, osteoclast numbers in retinoid-plus-CT–treated animals were about half of those found in rats given retinoid alone. Retinoid-induced hypercalcemia was abolished as in the previous experiment. In the curative protocol, osteoclast numbers were higher than in the preventive and were not influenced by CT. Plasma Ca was 0.4 mM higher in the curative than in the preventive group.

The acute hypocalcemic effect measured 2 h after a single injection of CT was present both after 3 d and 6 d, 2 IU/rat of CT producing a decrease in plasma Ca of 0.93 + 0.08 mM and 0.85±0.05 mM (mean±SEM, n = 3), respectively. These results suggest that osteoclasts recruited by the retinoid were sensitive to the hormone.

Discussion

Increased bone destruction has long been known to occur in vitamin A poisoning in animals and man (7–9). Whereas it should be possible to use naturally occurring vitamin A or retinoic acid to stimulate bone resorption, we found both compounds to be impractical. Dosage of vitamin A is difficult because it goes to liver stores before hypervitaminosis A develops (21), and retinoic acid produced necroses at the injection site when it was administered parenterally at the dose level required to induce hypercalcemia. Therefore, we used a new synthetic retinoid, one of the most potent retinoids currently available in terms of vitamin A activity (10, 11). Administration did not pose any problem.

The balance and ^{45}Ca kinetics experiments as well as chemical analysis of the bones demonstrate that the retinoid stimulated bone resorption. This effect was likely to be due to a direct action on bone because vitamin A has been shown to stimulate bone resorption in organ culture in vitro (22). In contrast, the changes in intestinal Ca absorption and in renal Ca excretion were probably indirect consequences of the increased Ca release from bone. Because plasma $1,25(OH)_2D$ was decreased by retinoid given at large dose and because this vitamin D metabolite is considered to be the main stimulator of intestinal Ca absorption (23), the retinoid-induced decrease of the latter could be mainly explained by the decrease in plasma $1,25(OH)_2D$. The decrease in plasma $1,25(OH)_2D$ could be due to a direct effect of the retinoid on the renal 25(OH)D-1-hydroxylase (24). However, in view of the close inverse correlation between plasma $1,25(OH)_2D$ and plasma Ca, it appears more likely that the retinoid influenced plasma $1,25(OH)_2D$ indirectly through its hypercalcemic effect. Several studies in the literature suggest a regulatory role of extracellular Ca in vitamin D metabolism (23, 25). An intermediate dose of retinoid produced a small but statistically significant decrease in intestinal Ca absorption without changing plasma Ca (Fig. 2). Therefore, an additional, possibly $1,25(OH)_2D$-independent effect of the retinoid on the intestine cannot be ruled out.

Our data provide no evidence for a renal effect of the retinoid on Ca metabolism. The retinoid-induced increase in urinary Ca is explained by an increase in filtered load as a consequence of hypercalcemia. Moreover, retinoid-induced hypercalcemia was completely prevented by Cl_2MBP. This inhibitor of bone resorption has been shown not to influence the renal handling of Ca (26), and therefore a renal contribution to the retinoid-induced hypercalcemia should have become apparent in Cl_2MBP-treated animals.

It appears, therefore, that the retinoid influenced Ca metabolism by a primary action on bone resorption, which led to the observed changes in plasma Ca, $1,25(OH)_2D$, Ca absorp-

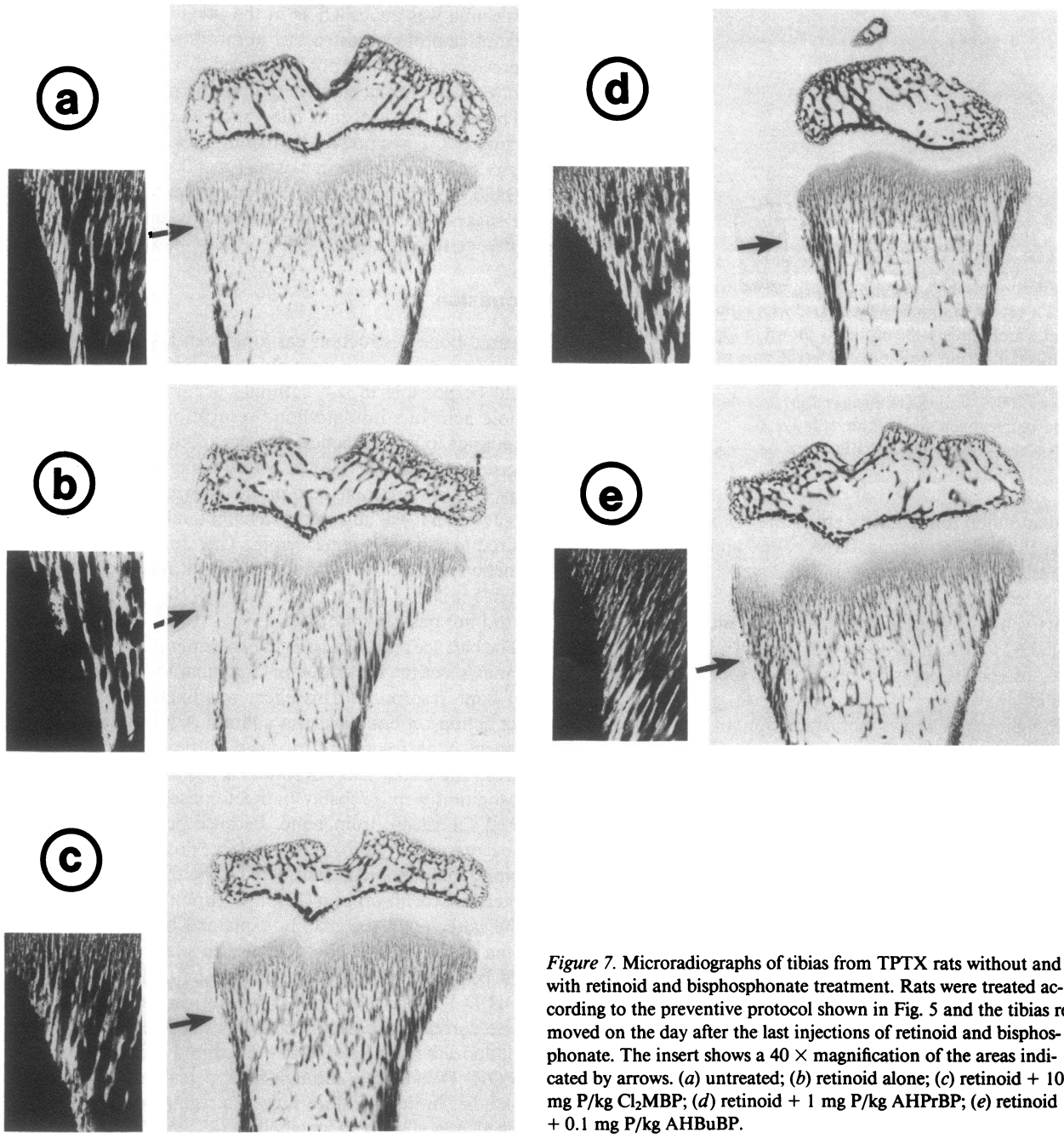


Figure 7. Microradiographs of tibias from TPTX rats without and with retinoid and bisphosphonate treatment. Rats were treated according to the preventive protocol shown in Fig. 5 and the tibias removed on the day after the last injections of retinoid and bisphosphonate. The insert shows a 40 × magnification of the areas indicated by arrows. (*a*) untreated; (*b*) retinoid alone; (*c*) retinoid + 10 mg P/kg Cl_2MBP; (*d*) retinoid + 1 mg P/kg AHPrBP; (*e*) retinoid + 0.1 mg P/kg AHBuBP.

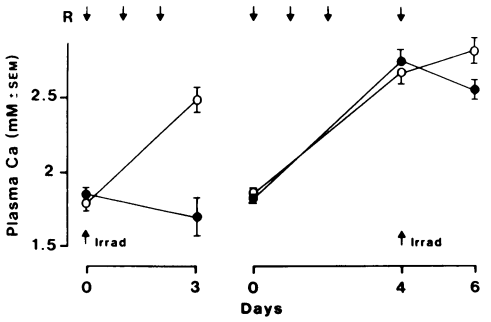


Figure 8. Effect of irradiation on retinoid-induced hypercalcemia in TPTX rats. All animals were given retinoid (*R*) at 25 μg/rat. Values are means±SEM, n = 4–5. (○) Retinoid; (●) retinoid and irradiation at time points indicated by arrows.

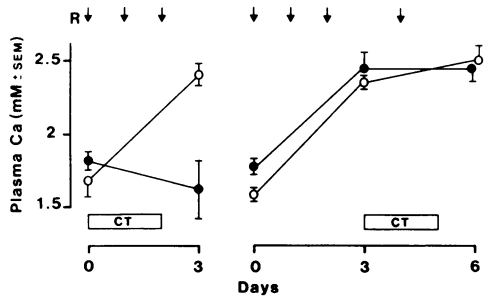


Figure 9. Effect of calcitonin (*CT*) on retinoid-induced hypercalcemia in TPTX rats. All animals were given retinoid (*R*) at 25 μg/rat. 8 IU/kg per d CT was given for 3 d as indicated. Values are means±SEM, n = 4–5. (○) Retinoid; (●) retinoid + CT.

Table IV. Effect of CT on Number of Osteoclasts in the Vertebral Periosteum of Retinoid-treated TPTX Rats

	Number of osteoclasts per millimeter of periosteal surface	
	Preventive protocol	Curative protocol
Retinoid	4.35±0.60	10.60±1.04
Retinoid + CT	2.29±0.59*	10.73±1.12

Experimental protocol was same as that of Fig. 9. Vertebrae were removed on day 3 in the preventive and on day 6 in the curative protocol. Untreated TPTX rats had 1.03±0.19 osteoclasts per mm of periosteal surface. Values are means±SEM; $n = 5$; $^*P < 0.05$.

tion, and urinary Ca. The increase in plasma Ca with retinoid was therefore a direct consequence of stimulated bone resorption, suggesting that this parameter could be used to monitor changes in bone resorption. Plasma Ca would not be the most sensitive parameter because an intermediate dose of retinoid which did produce a significant increase in Ca release from bone as assessed by ^{45}Ca kinetics (Fig. 2) had no effect on plasma Ca. However, as we subsequently demonstrated, plasma Ca can be very useful to monitor the effect of inhibitors of bone resorption in this model.

The treatment schedule shown in Fig. 5 was found to be optimal with respect to the rapidity of the increase in plasma Ca vs. the appearance of general signs of hypervitaminosis A, which occurred only after hypercalcemia was established. The observed decrease in bone mass demonstrated the stimulatory effect of the retinoid on bone destruction under these experimental conditions. Moreover, the finding of an increase in the number of osteoclasts suggested that the increased bone resorption was mainly due to the generation of new osteoclasts rather than an activation of those present in bone. An increase of osteoclast numbers has previously been reported with vitamin A in vivo (7, 9) and in vitro (22).

The effect of bisphosphonates on retinoid-induced hypercalcemia shows that plasma Ca is a reliable index for the effect of inhibitors of bone resorption in this model. These pharmacological agents have been shown to inhibit bone resorption specifically in vivo and in vitro (19). The dose-related inhibition of retinoid-induced hypercalcemia agrees very well with histomorphometric data obtained with these three compounds (20), the potencies of the bisphosphonates being identical with both methods. The compounds had similar potencies in preventing and reversing retinoid-induced hypercalcemia, showing that the sensitivity of bone resorption to this type of inhibitor was similar in both situations.

While the observed increase in the number of osteoclasts showed that recruitment of new osteoclasts was involved in retinoid-induced bone resorption, they did not indicate whether this occurred at the level of proliferation of osteoclast precursors or by an effect on the fusion of postmitotic precursors. To address this question and moreover to assess whether recruitment was also of importance in maintaining retinoid-induced bone resorption at a high level, the irradiation experiments were performed. The prevention of retinoid-induced hypercalcemia by irradiation suggests that proliferation of osteoclast precursors plays a major role in the stimulation of bone resorption. However, from the lack of effect of irradiation in reducing established hypercalcemia, it appears that proliferation of osteoclast precursors is not important in maintaining retinoid-induced bone resorption.

The results obtained with CT on retinoid-induced hypercalcemia are strikingly similar to those of the irradiation experiments, suggesting that this hormone inhibited the proliferation of osteoclast precursors. This conclusion is in agreement with reports in the literature which showed that CT inhibited the vitamin A–induced increase in osteoclast number in organ culture in vitro (22). A decrease of osteoclast number with CT given for 4 wk was also observed in vivo in TPTX rats (27). In the curative protocol, osteoclast numbers were higher than those in the preventive (Table IV), suggesting that new osteoclasts may have been formed between day 3 and 6 in the curative protocol. CT did not affect this process. Because the experiments with irradiation show that proliferation played a minor role in maintaining bone resorption at a high level in the 3-d period of the curative protocol studied, an increase in osteoclast number in this period appears to be due to a postmitotic process unaffected by CT. The acute hypocalcemic effect of CT in the preventive and curative protocols confirms the well-known direct inhibitory action of CT on osteoclasts (28).

A comparison of the effects of CT with those of bisphosphonates on retinoid-induced hypercalcemia demonstrates that this model is suitable to distinguish between different mechanisms of action of inhibitors of bone resorption. Whereas CT appears to inhibit proliferation of osteoclast precursors, at least one of the actions of bisphosphonates is independent of this process. However, it remains to be established whether they act on recruitment as well. A histological investigation is required to answer this question.

In conclusion, we have shown that retinoid-induced bone resorption represents a new animal model to study the induction of this process in vivo. Because proliferation of osteoclast precursors appears to play a major role in this system, the model may be a valuable tool to study this process in vivo. To our knowledge, no comparable animal model has yet been described. Moreover, the study shows that retinoid-induced bone resorption is suitable to study the effects of inhibitors of bone resorption, and, moreover, to differentiate between effects on the activity of osteoclasts present and on proliferation of osteoclast precursors. Because much information can be obtained by monitoring plasma Ca, the model represents also an efficient system to test new inhibitors of bone resorption and may be of value in the development of new compounds.

Acknowledgments

We wish to thank Ms. I. Boerlin, C. Engel, C. Marti, B. Rohrbach, and I. Tschudi for their excellent technical assistance, Ms. C. Stieger for her assistance with the illustrations, and Ms. B. Meier and B. Järmann for preparing the manuscript.

The work has been supported by the Swiss National Science Foundation (3.937.82).

References

1. Ibbotson, K. J., S. M. D'Souza, J. A. Kanis, D. L. Douglas, and R. G. G. Russell. 1980. Physiological and pharmacological regulation of bone resorption. *Metab. Bone Dis. & Relat. Res.* 2:177–189.

2. Raisz, L. G., and J. A. Lorenzo. 1980. Interactions of hormones,

ions, and drugs in the regulation of osteoclastic bone resorption. *Adv. Exp. Med. Biol.* 128:579–596.

3. Baron, R., A. Vignery, and M. Horowitz. 1984. Lymphocytes, macrophages and the regulation of bone remodeling. *In* Bone and Mineral Research. Ann. 2 W. A. Peck, editor. Elsevier, Amsterdam. 175–243.

4. Marks, S. C., Jr. 1983. The origin of osteoclasts: evidence, clinical implications and investigative challenges of an extra-skeletal source. *J. Oral Pathol.* 12:226–256.

5. Chambers, T. J. 1985. The pathobiology of the osteoclast. *J. Clin. Pathol. (Lond.).* 38:241–252.

6. Russell, R. G. G., R. C. Mühlbauer, S. Bisaz, D. A. Williams, and H. Fleisch. 1970. The influence of pyrophosphate, condensed phosphates, phosphonates and other phosphate compounds on the dissolution of hydroxyapatite in vitro and on bone resorption induced by parathyroid hormone in tissue culture and in thyroparathyroidectomised rats. *Calcif. Tissue Res.* 6:183–196.

7. Wolbach, S. B. 1947. Vitamin A deficiency and excess in relation to skeletal growth. *J. Bone Jt. Surg.* 29:171–192.

8. Bélanger, L. F., and I. Clark. 1967. Alpharadiographic and histological observations on the skeletal effects of hypervitaminoses A and D in the rat. *Anat. Rec.* 158:443–452.

9. Barnicot, N. A., and S. P. Datta. 1972. Vitamin A and bone. *In* The Biochemistry and Physiology of Bone. Vol. 2. G. H. Bourne, editor. Academic Press, New York. 197–229.

10. Loeliger, P., W. Bollag, and H. Mayer. 1980. Arotinoids, a new class of highly active retinoids. *Eur. J. Med. Chem. Chim. Ther.* 15:9–15.

11. Bollag, W. 1981. Arotinoids. A new class of retinoids with activities in oncology and dermatology. *Cancer Chemother. Pharmacol.* 7:27–29.

12. Aubert, J.-P., and G. Milhaud. 1960. Méthodes de mesure des principales voies du métabolisme calcique chez l'homme. *Biochim. Biophys. Acta.* 39:122–139.

13. Bonjour, J.-P., U. Trechsel, H. Fleisch, R. Schenk, H. F. DeLuca, and L. A. Baxter. 1975. Action of 1,25-dihydroxyvitamin D_3 and a diphosphonate on calcium metabolism in rats. *Am. J. Physiol.* 229:402–408.

14. Bonjour, J.-P., R. Rizzoli, K. Hugi, B. Haldimann, and H. Fleisch. 1978. Effect of synthetic vitamin D derivatives on calcium and phosphate metabolism in intact vitamin D-replete rats. *Metab. Bone Dis. & Relat. Res.* 1:23–28.

15. Hefti, E., U. Trechsel, H. Rüfenacht, and H. Fleisch. 1980. Use of dermestid beetles for cleaning bone. *Calcif. Tissue Int.* 31:45–47.

16. Bond, V. P., T. M. Fliedener, and J. O. Archambeau. 1965. Mammalian Radiation Lethality. Academic Press, New York.

17. Chen, P. S., Jr., R. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756–1758.

18. Trechsel, U., C. M. Taylor, J. A. Eisman, J.-P. Bonjour, and H. Fleisch. 1981. Plasma levels of vitamin D metabolites in diphosphonate-treated rats. *Clin. Sci. (Lond.).* 61:471–476.

19. Fleisch, H. 1983. Bisphosphonates: mechanism of action and clinical applications. *In* Bone and Mineral Research. Ann. 1. W. A. Peck, editor. Excerpta Medica, Amsterdam. 319–357.

20. Schenk, R., P. Eggli, H. Fleisch, and S. Rosini. 1986. Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. *Calcif. Tissue Int.* 38:342–349.

21. Goodman, D. S. 1984. Vitamin A and retinoids in health and disease. *N. Engl. J. Med.* 310:1023–1031.

22. Reynolds, J. J. 1968. Inhibition by calcitonin of bone resorption induced in vitro by vitamin A. *Proc. Roy. Soc. B.* 170:61–69.

23. DeLuca, H. F., and H. K. Schnoes. 1983. Vitamin D: recent advances. *Annu. Rev. Biochem.* 52:411–439.

24. Trechsel, U., and H. Fleisch. 1981. Retinoid and retinoic acid modulate the metabolism of 25-hydroxyvitamin D_3 in kidney cell culture. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 135:115–118.

25. Trechsel, U., J. A. Eisman, J. A. Fischer, J.-P. Bonjour, and H. Fleisch. 1980. Calcium-dependent, parathyroid hormone-independent regulation of 1,25-dihydroxyvitamin D. *Am. J. Physiol.* 239:E119–E124.

26. Troehler, U., J.-P. Bonjour, and H. Fleisch. 1982. Interference of dichloromethane diphosphonate with parathyroid hormone effects at the bone but not at the kidney level. *Miner. Electrolyte Metab.* 7:122–126.

27. Foster, G. V., F. H. Doyle, P. Bordier, and H. Matrajt. 1966. Effect of thyrocalcitonin on bone. *Lancet.* ii:1428–1431.

28. Talmage, R. V., C. W. Cooper, and S. V. Toverud. 1973. The physiological significance of calcitonin. *In* Bone and Mineral Research. Ann. 1. W. A. Peck, editor. Excerpta Medica, Amsterdam. 74–143.