

Formation, Mineralization, and Resorption of Bone in Hypophosphatemic Rats

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ABSTRACT Quantitative morphologic methods were used to measure the effects of feeding a low phosphorus diet to intact and thyroparathyroidectomized rats on several processes of bone mineralization and turnover. In severely hypophosphatemic animals, the matrix formation rate was decreased, the osteoid maturation rate was decreased, which indicated a delay in the onset of mineralization, the initial rate of mineralization was decreased, and the endosteal osteoclastic bone resorption rate was increased. In moderately hypophosphatemic animals, there was a substantial increase in bone resorption but no change in formation or in mineralization. The increase in endosteal bone resorption was due to an increase in the linear rate of bone resorption and particularly to an increase in the length of the endosteal resorbing surface. The magnitude of the increase in bone resorption was similar in thyroparathyroidectomized and intact rats indicating that neither parathyroid hormone nor calcitonin is involved in this change. This, together with the finding that there was a strong negative correlation ($r = -0.99$) between the per cent endosteal resorbing surface and the serum phosphorus, supports the view that the increased resorption was due to hypophosphatemia. This inverse relationship between endosteal resorbing surface and serum phosphorus appeared to hold for values of serum phosphorus above normal. The resorptive response to hypophosphatemia, as previously shown for the resorptive response to excess endogenous parathyroid hormone, was partially inhibited by vitamin D deficiency. Increased resorption occurred at levels of serum phosphorus where no changes were observed in bone formation, mineralization, or growth, suggesting that this resorptive response functions as a homeostatic mechanism to maintain serum and intracellular phosphorus concentrations.

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INTRODUCTION

Numerous studies in the old as well as in the more recent literature establish that hypophosphatemia has important effects on bone metabolism (1-5). Raisz, using tissue culture methods, has recently demonstrated a direct relationship between matrix formation and the level of phosphorus in the medium (5). Phosphorus-depleted experimental animals (2) and human subjects (3) develop impairment of mineralization as manifested by rickets or osteomalacia. A low phosphorus diet fed to rats results in enlargement of the medullary cavity (1), a finding suggestive of increased endosteal osteoclastic resorption. Further support for the concept that hypophosphatemia stimulates bone resorption is found in the studies by Raisz and Niemann showing that in tissue culture a reduction of the level of phosphorus in the incubation medium is associated with increased resorption (6).

However, these past studies provide little information on the effects of hypophosphatemia on the specific processes involved in matrix formation and mineralization and bone resorption, and changes in rates of these processes due to hypophosphatemia have not been measured directly in vivo. Furthermore, the nature of the impairment of mineralization as a result of hypophosphatemia has not been established. The over-all process of mineralization can be divided into two stages: the initiation of mineralization and the increase in mineral concentration once mineralization is initiated at the mineralizing front (7). Although neither of these two stages has been quantitated in the presence of hypophosphatemia, past studies showing that osteoid width is increased in hypophosphatemic animals (2) and that mineral deposition may be increased in the presence of hyperphosphatemia, (8) suggest that the level of serum phosphorus may be rate limiting in both of these processes.

The present study was undertaken to define more clearly the effects of hypophosphatemia on bone. The effects of low to normal dietary phosphorus levels on the above mentioned processes were measured in the rat by means of quantitative morphologic techniques.

METHODS

Because a detailed treatment of the methods used in this study has been given elsewhere (7, 9, 10), only a brief description of the parameters relating to the processes of formation, mineralization, and resorption will be presented. The method used to measure the rates of formation and resorption takes advantage of the fact that in the tibial diaphysis of growing rats there is a progressive increase in bone area due to bone formation and a progressive increase in medullary cavity area due to bone resorption (10). During the measuring period all rats are injected daily with tetracycline¹ so that all new bone formed during this period can be identified by its tetracycline fluorescence.

Bone parameters

Bone formation rate (cubic millimeters per day). This is the amount of mineralized bone formed at the periosteum or endosteum per day. It is determined from the amount of bone labeled with tetracycline during the measuring period. The bone formation which occurs around vascular canals and which makes up about 10% of the bone formation occurring in our sampling site (10) was not measured in this study.

Matrix formation rate (cubic millimeters per day). This includes all matrix formed, regardless of whether or not it mineralizes during the measuring period. It is usually identical with the bone formation rate except when there is a mineralization defect such that the amount of osteoid formed is greater than the amount mineralized during the measuring period.

Bone apposition rate (microns per day). This is the width of mineralized bone added per day at a bone forming surface and is calculated by dividing the amount of bone formed by the length of the forming surface.

Matrix apposition rate (microns per day). This is the width of new matrix added per day and includes both osteoid and mineralized matrix.

Osteoid maturation rate (per cent per hour). This is a measure of the onset of mineralization. A certain amount of time elapses between the deposition of osteoid and the initiation of mineralization in this osteoid. This time can be calculated by dividing the width of osteoid by the matrix apposition rate. If one assumes that osteoid is 0% mature when it is formed and 100% mature when mineralization is initiated, the above time is the time to reach 100% maturity. Therefore, the osteoid maturation rate is calculated simply by dividing 100% by the above time.

This calculation makes no assumptions about the nature of the changes which must occur before mineralization can be initiated. The term "maturation" is used because we have demonstrated that chemical changes do occur in osteoid before the onset of mineralization (11).

A previous study demonstrating a direct relationship between osteoid width and matrix apposition (7) suggests that osteoid maturation rate is probably a better measurement of the onset of mineralization than is osteoid width. Accord-

ingly, an increase in osteoid width could be related to either an increase in apposition rate or a delay in the onset of mineralization whereas the osteoid maturation rate is independent of apposition.

Initial mineralization rate (per cent of maximum per hour). In previous studies we demonstrated that after injection tetracycline diffuses into bone which has attained about 20% of its maximum mineral concentration, but no further. The time required to reach 20% of maximum mineralization is determined by converting the width of this initial band of tetracycline uptake to time, using the relationship given by the bone apposition rate. The mineral concentration divided by this time yields this rate.

Endosteal bone resorption rate (cubic millimeters per day). Since no resorption occurs at the periosteum in our sampling site in the tibial diaphysis, this represents essentially the total resorption rate. A small amount of resorption occurs around vascular canals, but this is only about 10% of the total (10) and is ignored. The endosteal bone resorption rate is equal to the change in medullary area plus the endosteal bone formation rate (7, 9, 10). The endosteal bone formation rate is determined by the tetracycline-labeling procedure, and the change in medullary area during the experimental period is determined by sacrificing one group of animals at the beginning of the experiment for measurements of basal medullary area and subtracting this from the medullary area found at the end of the experiment in another group of animals.

Linear rate of endosteal bone resorption (microns per day). This is the mean width of endosteal bone resorbed per day and is calculated by dividing the total volume of bone resorbed at the endosteum by the length of the endosteal-resorbing surface. It is analogous to the bone apposition rate.

Diets

The semisynthetic diet used in this work has been described elsewhere (12). The diet used for intact control animals contains 0.6% calcium and 0.6% phosphorus. By varying the concentrations of salts (potassium phosphate, calcium carbonate, and potassium chloride) added to the diet, we obtained diets with final calcium concentrations from 0.6 to 1.2%, with final phosphorus concentrations from 0.2 to 0.6% and with a constant potassium concentration. Casein, the dietary protein source, supplies 0.2% phosphorus to the diet. In order to study the effects of a very low concentration of phosphorus in the diet, fibrin instead of casein was used as the source of protein, and the amount of phosphorus in the fibrin was such that with the phosphate salts omitted, the final concentration of phosphorus in the diet was 0.04%.

Serum chemistries

Blood was obtained by cardiac puncture at sacrifice or from a tail vein when a series of determinations were made on the same animals. Total serum calcium and phosphorus determinations were made as previously described (7), and ionized calcium was measured on freshly obtained serum by means of an Orion model 98-20 flow-through electrode system (Orion Research, Inc., Cambridge, Mass.) (13).

Experiments

(1) *Effects of a 0.2% phosphorus diet on intact rats.* Weanling rats were divided into 3 groups of 12 animals each. One group was injected i.v. with 20 mg/kg of tetracycline and sacrificed 2 hr later for base line measurements of bone parameters. The other two groups were injected i.p. with

¹ Demethylchlortetracycline, kindly supplied by Dr. Hugh McDonald, Lederle Laboratories, Pearl River, N. Y.

TABLE I
Serum and Bone Parameters in Intact and TPTX Rats Fed Control or Low Phosphorus Diets

	Intact		TPTX	
	0.6% P	0.2% P	0.55% P	0.25% P
Body weight, g	98.1 ± 4.1*	86.0 ± 4.7‡	100.7 ± 7.3	85.1 ± 7.0‡
Serum Ca, mg/100 ml	9.9 ± 0.3	11.1 ± 0.5‡	10.0 ± 0.9	11.2 ± 0.4‡
Serum P, mg/100 ml	10.3 ± 1.1	6.2 ± 0.8‡	11.5 ± 1.2	7.3 ± 1.1‡
Serum Ca × P product, (mg/100 ml) ²	104 ± 11	69 ± 8‡	117 ± 17	81 ± 12‡
Periosteal osteoid width, μ	7.9 ± 1.0	12.1 ± 1.4‡	8.7 ± 2.2	9.3 ± 1.2
Periosteal mineral front width, μ	5.2 ± 0.5	5.6 ± 0.5	4.9 ± 0.9	5.1 ± 0.9
Total area, mm ² §	2.66 ± 0.19	2.57 ± 0.10	2.67 ± 0.20	2.63 ± 0.18
Medullary area, mm ²	0.99 ± 0.15	1.43 ± 0.13‡	0.86 ± 0.11	1.04 ± 0.10‡
Periosteal surface, mm	5.85 ± 0.17	5.67 ± 0.27	6.05 ± 0.21	5.93 ± 0.20
Endosteal forming surface, mm	2.27 ± 0.54	0.25 ± 0.28‡	1.61 ± 0.47	0.52 ± 0.39‡
Endosteal resorbing surface, mm	1.51 ± 0.55	4.23 ± 0.31‡	2.00 ± 0.46	3.63 ± 0.49‡
Endosteal resorbing surface, %	39.8 ± 12.5	94.6 ± 6.2‡	55.4 ± 12.8	87.4 ± 9.4‡

* Mean ± SD.

‡ $P < 0.001$, probability estimated by t test.

§ Total area is the area circumscribed by the periosteal border of bone and includes the medullary cavity.

tetracycline daily starting at the time that the basal group was sacrificed and continuing until 2 days before sacrifice. At the time the tetracycline injections were started, the level of dietary phosphorus in the experimental group was reduced to 0.2%. The control rats were pair fed with the experimental group. 11 days after starting the tetracycline injections, the two groups were sacrificed 2 hr after a final i.v. injection of tetracycline, and the tibias were removed for analysis. The final tetracycline injection was given to determine the final mineralizing front width, which is needed to calculate the mineralization rate (7).

(2) *Effects of a 0.04% phosphorus diet on intact rats.* Weanling rats were divided into two groups of eight animals each. At the beginning of the experimental period, all rats were injected subcutaneously with 15 mg/kg of tetracycline. At this time, the experimental group was given a diet containing 0.6% calcium and 0.04% phosphorus with fibrin as the source of protein, and the control group was given the same diet except that sufficient potassium phosphate was added so that the phosphorus content was 0.6%. The control group was pair weighted with the experimental group. 14 days later all rats were sacrificed, and the tibias were removed for analysis. Body weight was determined after removal of stomach and intestines to avoid the effect of differences in gut contents on body weight. The basal values obtained in experiment 1 were used to calculate the bone resorption rate in experiment 2 since the rats in both experiments were the same age at the beginning of the experiment. Because of this, the values for the bone resorption rate in this experiment are probably not as reliable as those in the other experiments.

(3) *Varying dietary phosphorus levels on thyroparathyroidectomized (TPTX)² rats.* Using ether anesthesia, weanling rats were TPTX by blunt dissection. The rats received a diet containing 0.6% calcium and 0.6% phosphorus and were injected every other day with 4 μg of L-thyroxin (Levoid; Nutrition Control Products, Hollywood, Fla.). 3 days after TPTX, blood samples were drawn from the tail for

² Abbreviation used in this paper: TPTX, thyroparathyroidectomized.

serum calcium analysis. Rats with serum calcium values of less than 9.0 mg/100 ml were divided into groups of five or six animals each. Each group was then started on a diet containing 1.2% calcium and from 0.25 to 0.6% phosphorus. 10 days later the rats were sacrificed, and the tibias were removed for analysis.

(4) *Effects of a 0.25% phosphorus diet on TPTX rats.* TPTX rats were divided into 3 groups of 10–15 animals each. The first group was sacrificed 2 hr after an i.p. injection of 10 mg/kg of tetracycline for base line measurements of bone parameters. At this time, the control group was given a 1.2% calcium and 0.55% phosphorus diet, and the experimental group was given a 1.2% calcium and 0.25% phosphorus diet. Both groups were injected i.p. every 3rd day with tetracycline starting at the time the basal group was sacrificed. 10 days later and 2 hr after the last i.p. injection of tetracycline, the rats in the two final groups were sacrificed, and the tibias were removed for analysis.

(5) *Effects of length of depletion period.* Groups of TPTX rats, six rats per group, received a diet containing either 0.55 or 0.30% phosphorus and 1.2% calcium. At 0, 1, 2, 4, and 10 days, one group from each dietary level of phosphorus was sacrificed, and the tibias were removed for analysis.

(6) *Effects of a diet containing 0.25% phosphorus and no vitamin D.* 3 groups of TPTX rats, 12 rats per group, were used. After TPTX, all groups were given a diet containing 0.55% phosphorus and 1.2% calcium, but in one group vitamin D was omitted from the diet. When the rats were 33 days of age, the dietary phosphorus was reduced to 0.25% in the vitamin D-deficient and one other group, and 10 days later all groups were sacrificed, and the tibias were removed for analysis.

RESULTS

A low phosphorus diet (0.2%) fed to intact rats resulted in hypophosphatemia and hypercalcemia (Table I). In another experiment we found in hypophosphatemic rats that the serum ionized calcium was increased and that

TABLE II
Serum Phosphorus, Calcium, and Ionized Calcium in Intact Rats Fed Normal or Low Phosphorus (0.04%) Diets*

	Control	Low phosphorus
Serum phosphorus, mg/100 ml	9.8 ± 0.5‡	5.0 ± 1.0§
Serum calcium, mg/100 ml	10.5 ± 0.2	13.8 ± 0.6§
Serum ionized calcium, mg/100 ml	5.3 ± 0.2	7.0 ± 0.4§

* Measurements were made 3 days after starting the diets.

‡ Mean ± SD.

§ $P < 0.001$.

the increase in this fraction contributed to the increase in total serum calcium (Table II).

In intact hypophosphatemic rats there were a number of changes in bone, the most striking of which were related to the process of endosteal bone resorption. The length of the endosteal resorbing surface was increased,

and the medullary area was enlarged due to an increase in the rate of endosteal bone resorption (Tables I and III). The increase in endosteal resorbing surface was not simply due to an increase in total endosteal surface because the amount of forming surface decreased in the experimental group during the experiment, indicating that at least part of the increase in resorbing surface was due to a conversion of forming surface to resorbing surface.

Changes also were found in matrix formation and mineralization (Table III). There were statistically significant decreases in the rates of both periosteal matrix formation and periosteal matrix apposition. However, despite similar food intakes, the hypophosphatemic group weighed less than the control group at the end of the experiment, and the decrease in final body weight was similar to the decrease in periosteal and in total (periosteal plus endosteal) matrix formation (Tables I and III). Thus, the decrease in bone formation may have been part of a generalized inhibition of growth rather than a specific effect of the low phosphorus diet on bone formation. In the hypophosphatemic group, there was an increase in osteoid width which resulted

TABLE III
Bone Matrix Formation, Mineralization, and Resorption in Intact and TPTX Rats Fed Control or Low Phosphorus Diets

	Intact		TPTX	
	0.6% P	0.2% P	0.55% P	0.25% P
Periosteal bone formation rate, mm ³ /day	0.067 ± 0.008*	0.056 ± 0.005‡	0.057 ± 0.010	0.055 ± 0.001
Periosteal matrix formation rate, mm ³ /day	0.068 ± 0.008	0.059 ± 0.006‡	0.057 ± 0.010	0.055 ± 0.011
Endosteal matrix formation rate, mm ³ /day	0.008 ± 0.002	0.001 ± 0.001§	0.007 ± 0.006	0.001 ± 0.001§
Total matrix formation rate, ¶ mm ³ /day	0.076 ± 0.009	0.060 ± 0.005§	0.064 ± 0.010	0.055 ± 0.012
Periosteal matrix apposition rate, μ/day	12.5 ± 1.3	11.1 ± 1.2**	9.9 ± 1.7	9.7 ± 1.8
Periosteal osteoid maturation rate, %/hr	6.70 ± 0.93	4.65 ± 0.48§	4.25 ± 0.80	4.02 ± 0.74
Periosteal initial mineralization rate, % of maximum/hr	2.27 ± 0.28	1.87 ± 0.20§	1.10 ± 0.23	1.02 ± 0.20
Endosteal bone resorption, mm ³ /day	0.027 ± 0.009	0.060 ± 0.006§	0.014 ± 0.008	0.027 ± 0.007§
Linear bone resorption rate, μ/day	13.4 ± 5.2	17.4 ± 1.5**	6.0 ± 3.5	8.3 ± 2.0**

* Mean ± SD.

‡ $P < 0.01$.

§ $P < 0.001$.

|| The rates of endosteal bone formation are underestimated in the low phosphate groups because some forming surface was converted to resorbing surface and as a result a portion of tetracycline-labeled bone was resorbed. For example, in the low phosphorus group of the TPTX study, since maximum endosteal label width was reduced 30% and since mean endosteal forming surface during the measuring period was reduced 40%, the true reduction in endosteal bone formation was about 60% as opposed to the 85% indicated by the rates in the Table.

¶ Total indicates both periosteal and endosteal formation.

** $P < 0.05$.

TABLE IV
Serum and Bone Parameters in Intact Rats Fed Control or Low Phosphorus (0.04%) Diets

			Control vs. low phosphorus	
	Control	Low phosphorus	% Change	P*
Body weight, g	59 ±4.0†	57 ±4.8	-3	NS
Serum Ca, mg/100 ml	9.4 ±0.3	11.2 ±0.5	19	<0.001
Serum P, mg/100 ml	10.2 ±0.8	5.1 ±0.9	-50	<0.001
Serum Ca × P product, (mg/100 ml) ²	96 ±7	57 ±11	-40	<0.001
Periosteal osteoid, μ	9.3 ±1.2	19.8 ±3.4	113	<0.001
Endosteal resorbing surface, %	51.0 ±7.8	96.7 ±3.6	89	<0.001
Medullary area, mm ²	0.93 ±0.10	1.54 ±0.11	66	<0.001
Cortical thickness, mm	0.34 ±0.02	0.14 ±0.01	-58	<0.001
Periosteal matrix formation rate, mm ³ /day§	0.040 ±0.007	0.020 ±0.007	-50	<0.001
Periosteal matrix apposition rate, μ/day§	7.7 ±1.4	3.2 ±0.4	-59	<0.001
Periosteal osteoid maturation rate, %/hr	3.73 ±0.53	0.96 ±0.12	-74	<0.001
Periosteal initial mineralization rate, % maximum/hr	1.38 ±0.36	0.35 ±0.05	-75	<0.001
Endosteal bone resorption rate, mm ³ /day	0.011 ±0.004	0.050 ±0.029	342	<0.001

* Probability estimated by *t* test.

† Mean ±SD.

§ Because evidence of bone resorption at the periosteum was found in some of the animals in the experimental group, only those animals which had less than 5% of the periosteal surface involved in resorption (i.e. five out of the eight animals in the experimental group) were used to calculate periosteal matrix formation. On the other hand, periosteal matrix apposition was measured in all eight animals in five equidistant positions where no periosteal resorption was occurring. This measurement was made in the same five positions in the control group.

from a delay in the onset of mineralization as evidenced by the reduction in the rate of osteoid maturation. The initial rate of mineralization was also reduced (Table III).

In order to determine if more severe phosphorus depletion would result in a decrease in matrix formation unrelated to weight gain and a greater inhibition of mineralization, rats were fed a lower (0.04%) phosphorus diet, and the control group was pair weighted with the experimental group. These rats developed more severe hypophosphatemia and more marked changes in all bone parameters (Table IV and Fig. 1). As can be seen in Table IV these severely hypophosphatemic animals had a marked decrease in periosteal matrix formation. In addition, osteoid width was increased 113% (Fig. 2), a change which in our experience is similar in magnitude to that seen in patients with phosphate diabetes.

The changes in bone resorption were also greater in this experiment. There was a 342% increase in endosteal bone resorption rate and as a result a marked increase in medullary area (Fig. 1). Almost the entire endosteal surface was involved in active bone resorption and even a portion of the periosteal forming surface was converted to resorbing surface in five of the eight rats in the experimental group, whereas normally in our sampling site all of the periosteal surface is involved in formation.

Because of the increased endosteal resorption and the decreased periosteal formation, cortical thickness was markedly decreased (Table IV, Fig. 1).

A number of experiments were done to explore further the relationship between the level of dietary phosphorus and the process of endosteal bone resorption. TPTX rats were studied to determine if the increased bone resorption would occur in the absence of parathyroid hormone and calcitonin. The ideal control for such an experiment would be a TPTX rat with normal levels of calcium and phosphorus. A preliminary experiment was done to determine if, by varying the level of phosphorus intake, we could obtain normal levels of serum calcium and phosphorus in TPTX rats. Fig. 3 shows that the serum phosphorus level rose with the increase in dietary phosphorus level and that there was a reciprocal relationship between the serum calcium and phosphorus. By interpolation, a diet containing 1.2% calcium and 0.55% phosphorus should result in normal levels of both serum calcium and phosphorus in TPTX rats.

When this diet was fed to TPTX control rats, they did in fact have normal levels of serum calcium and phosphorus, whereas rats fed a 0.25% phosphorus diet had hypophosphatemia in association with marked increases in the parameters relating to bone resorption (Tables I and III). Thus, the low phosphorus diet resulted in a

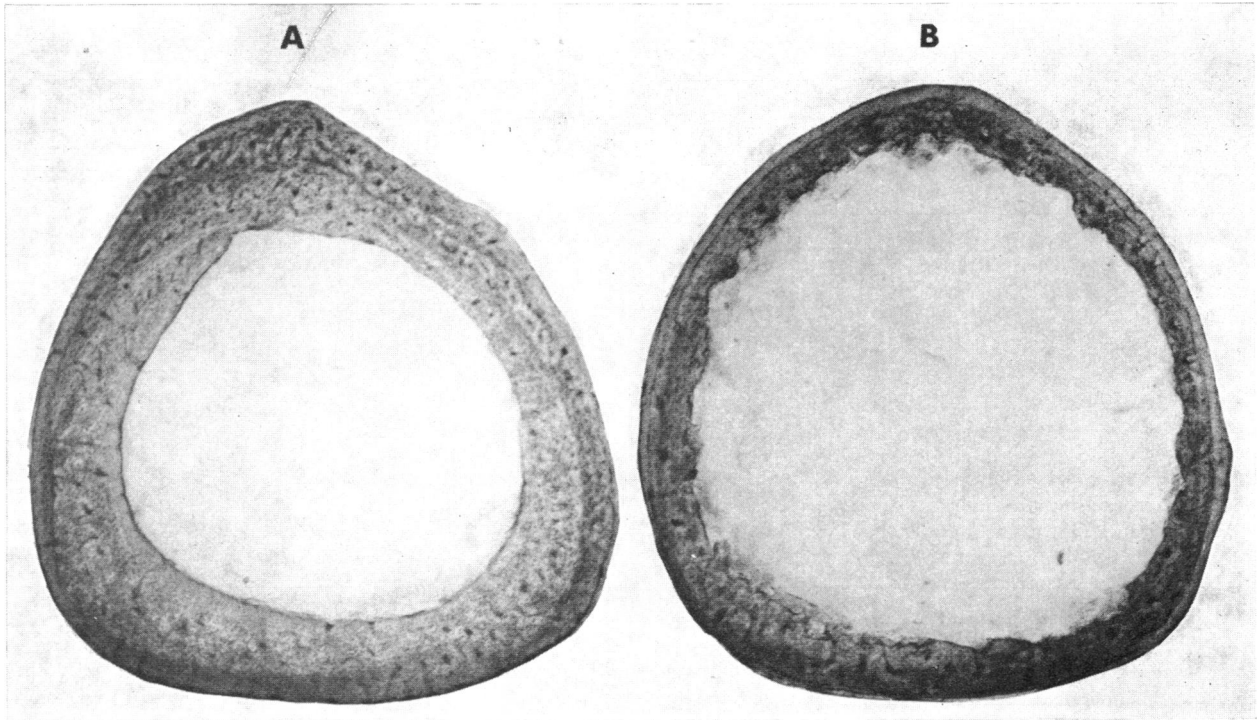


FIGURE 1 Bright-field photomicrographs of cross sections of the tibial diaphysis on the 14th day of the experimental period from (A) a pair-weighted control rat and (B) a rat fed a 0.04% phosphorus diet. The medullary area is increased, and the cortical thickness is decreased in the test animal. $\times 25$.

stimulation of bone resorption even in the absence of the parathyroid and thyroid glands. In contrast with intact hypophosphatemic rats, the decreases in bone formation and mineralization were not statistically significant in the TPTX hypophosphatemic rats, possibly because the dietary level of phosphorus was slightly higher in the TPTX than in the intact experimental group (Table III). Thyroparathyroidectomy per se does not reduce bone formation³ and therefore does not explain the lack of change in this experiment.

In TPTX rat fed diets containing from 0.25% to 0.6% phosphorus, there was an inverse relationship between the dietary level of phosphorus and two functions of endosteal osteoclastic bone resorption: the length of the endosteal resorbing surface and the medullary area (Fig. 4, top). Evidence of increased resorption was found at a dietary phosphorus level (0.4%) which did not cause changes in either osteoid width or in three growth parameters: tibial length, total area, and body weight (Fig. 4).

The relationship between serum phosphorus, ranging from 5 to 15 mg/100 ml and per cent endosteal resorbing

³ Baylink, D., J. Wergedal, M. Stauffer, and C. Rich. To be published.

surface is shown in Fig. 5. In the four TPTX groups given different levels of dietary phosphorus, there was a strong negative correlation ($r = -0.99$, $P < 0.001$) between these two parameters. As can be seen, the change in per cent endosteal resorbing surface as a function of serum phosphorus appeared to be of similar magnitude in TPTX and intact rats. In another group of TPTX rats with a mean serum phosphorus well above the normal level of 10 mg/100 ml, the per cent endosteal resorbing surface was considerably less than normal, suggesting that the inverse relationship between these two parameters holds for serum phosphorus values above normal.

When groups of TPTX rats were sacrificed after varying periods of phosphorus depletion, hypophosphatemia was evident 24 hr after the low phosphorus diet was started whereas an increase in endosteal resorbing surface was not found until at least 4 days on this regimen (Fig. 6). Bone resorption may have been increased by an increase in linear rate at an earlier time than the increase in resorbing surface, and the lack of a detectable change in medullary area (Fig. 6) does not rule this out. According to our calculations, a doubling of the linear rate of bone resorption, a response greater than that observed in a different experiment under similar

conditions, would not produce a statistically significant increase in medullary area by 4 days.

In previous studies we showed that the resorptive response to increased endogenous parathyroid hormone as a result of hypocalcemia in intact rats was partially inhibited by vitamin D deficiency (7). Therefore, an experiment was done to determine if vitamin D deficiency would also inhibit the resorptive response to hypophosphatemia. In this experiment there were three groups of TPTX rats: (a) control, (b) low dietary phosphorus, and (c) low dietary phosphorus and vitamin D deficient. As expected from the results shown in Table III, the low dietary phosphorus group had lower serum phosphorus and higher per cent endosteal resorbing surface than the control group (Fig. 7). As compared with the low dietary phosphorus group, the low dietary phosphorus and vitamin D-deficient group had an even lower serum phosphorus but a lower rather than higher per cent endosteal resorbing surface. Nevertheless, the per cent endosteal resorbing surface was greater in the low dietary phosphorus and vitamin D-deficient group than in the control group, indicating that vitamin D deficiency only partially inhibited the expected increase in the per cent endosteal resorbing surface.

DISCUSSION

The results of this study show that in rats with moderate hypophosphatemia there is a significant increase in osteoclastic bone resorption but no change in matrix formation or mineralization, whereas in rats with severe hypophosphatemia, there is a marked stimulation of bone resorption and, as well, a marked inhibition of matrix formation and mineralization (Tables III and IV).

Raisz found a marked reduction in the incorporation of radioprolin into bone matrix in tissue culture when the phosphorus concentration in the medium was reduced from about 13 to 6 mg/100 ml (5). This is consistent with our finding that when the serum phosphorus was markedly reduced from about 10 to 5 mg/100 ml, there was a marked reduction in periosteal matrix formation (Table IV). However, when the serum phosphorus was reduced from about 10 to 7 mg/100 ml (i.e. when the dietary phosphorus level was reduced from 0.6% to 0.4%), total area, which is a measure of periosteal bone formation, was unchanged (Figs. 3 and 4). Thus, in this work, formation remained normal until the serum phosphorus fell below about 7 mg/100 ml, whereas further decrements caused a sharp inhibition of formation.

Some years ago Freeman and McLean reported that

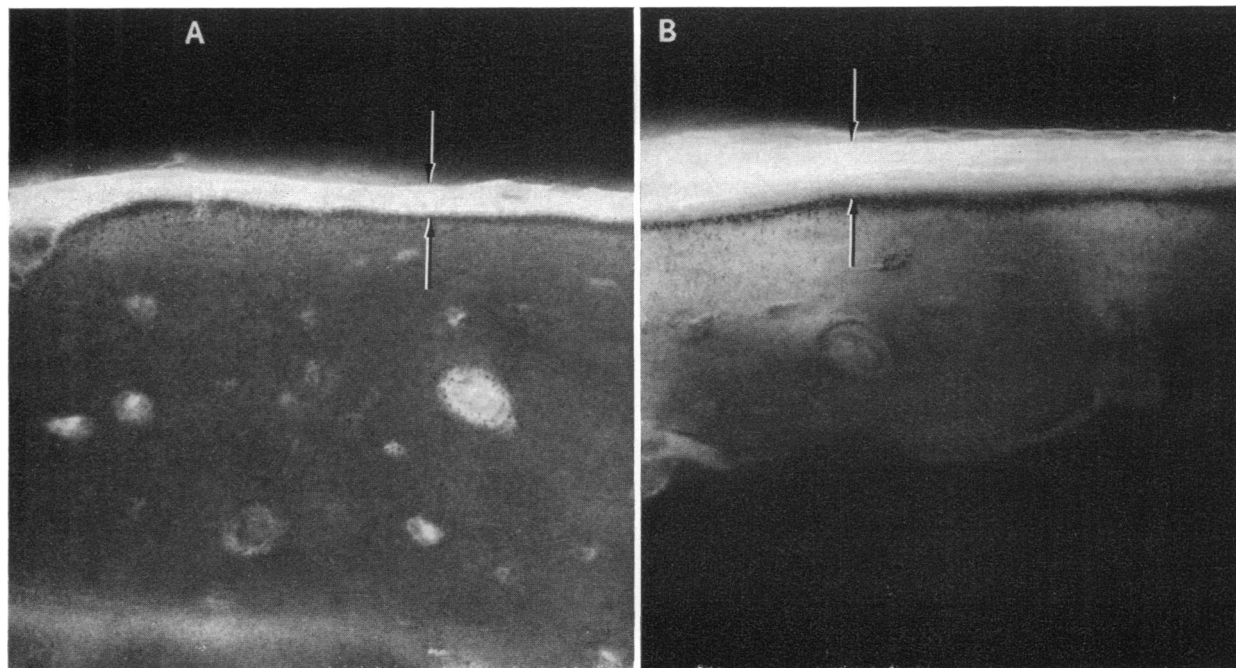


FIGURE 2 Fluorescence photomicrographs of periosteal osteoid on the 14th day of the experimental period from (A) a pair-weighted control rat and (B) a rat fed a 0.04% phosphorus diet. Osteoid width, as indicated by the arrows, is considerably increased in the test animal. $\times 315$.

phosphorus-depleted animals develop wide osteoid seams (2). We have confirmed this observation and, in addition, have shown that this is the result of a delay in the onset of mineralization as indicated by the finding that the osteoid maturation rate was decreased (Tables III and IV). Studies from this and other laboratories indicate that osteoid undergoes a number of chemical modifications or "matures" before mineralization (7, 11). We have interpreted this to indicate that cell-mediated processes are essential to the initiation of mineralization, and accordingly, the findings in this study suggest that severe hypophosphatemia inhibits these cell-mediated changes. Accompanying this inhibition of osteoid maturation was a comparable inhibition of the initial rate of mineralization. This observation is of particular interest because all of the other variables that we have studied thus far, including vitamin D deficiency (7), calcium deficiency (14), and fluoride intoxication (9), also have been found to cause an inhibition of both rates, suggesting that these two rates share common rate-limiting factors.

One of the most significant findings in hypophosphatemic rats was the marked stimulation of osteoclastic bone resorption, a finding which is consistent with our previous observation that acid β -glycerophosphatase is increased in bone homogenates from hypophosphatemic rats (15). In our sampling site, all of the periosteal surface is normally involved in formation and even when the endosteal resorption rate was increased 440% in rats fed a calcium-free diet, none of the forming surface at the periosteum was converted to resorbing surface.⁴ However, in hypophosphatemic rats, the intensity of the resorptive stimulus was so great in rats fed the 0.04% phosphorus diet that a portion of the periosteal surface became involved in resorption. Although the number of osteoclasts was not counted, it is difficult to envision that the observed large increase in endosteal-resorbing surface occurred without an increase in the number of osteoclasts. The increase in the volume of bone resorbed was not entirely due to increased osteoclastic proliferation, for we also observed an increase in the linear rate of bone resorption which is a measure of the resorptive activity per osteoclast.

Our finding that the magnitude of the increase in bone resorption was similar in intact and TPTX rat fed similar levels of dietary phosphorus indicates that parathyroid hormone and calcitonin were not involved in this response (Table III). This, together with the finding that there was a high negative correlation between serum phosphorus and per cent endosteal-resorbing surface, is consistent with the conclusion that hypophosphatemia was responsible for the increase in bone resorption

⁴ Stauffer, M., D. Baylink, J. Wergedal, and C. Rich. To be published.

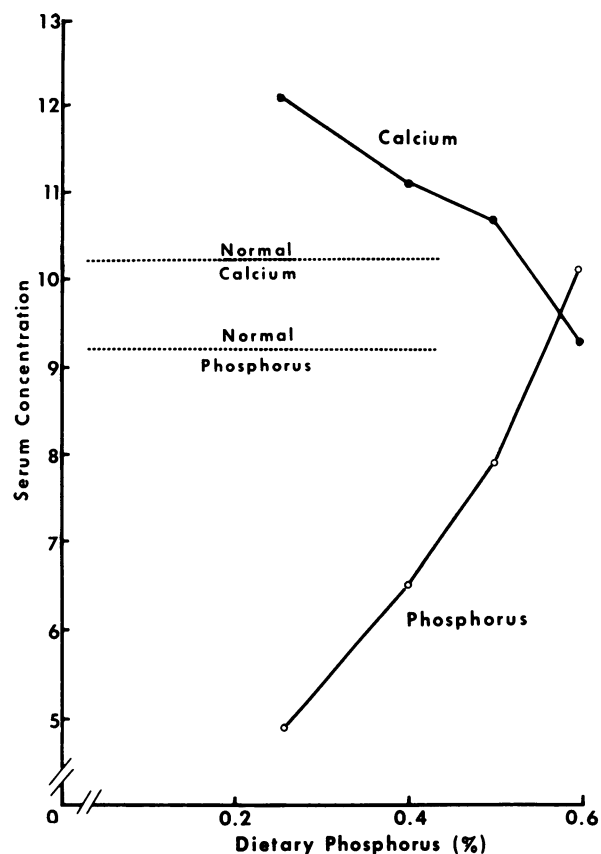


FIGURE 3 Effect of different levels of dietary phosphorus with a constant level of dietary calcium (1.2%) on the serum calcium and phosphorus in groups of TPTX rats. Each point represents a mean value from five to six rats. The indicated mean normal values for serum calcium and phosphorus were obtained from intact rats fed a normal diet containing 0.6% calcium and 0.6% phosphorus.

(Fig. 5). This conclusion is also supported by in vitro studies showing that bone resorption is enhanced by lowering the concentration of phosphorus in the medium (6, 16). Although hypercalcemia, which is known to stimulate cell proliferation (17), was present in these studies, it does not seem a likely explanation for the observed osteoclastic proliferation, in view of the experiments of Raisz and Niemann (6) showing that the resorptive response to a low level of phosphorus in the medium is reduced by increasing the level of calcium in the medium.

When animals were placed on a low dietary phosphorus regimen, the endosteal-resorbing surface was not demonstrably increased for at least 4 days even though hypophosphatemia was evident after 1 day. This strongly suggests that the effect of hypophosphatemia to stimulate osteoclastic proliferation is not immediate. However, the observation that hypercalcemia was also

apparent at 1 day may mean that hypophosphatemia causes an immediate increase in the transfer of calcium from bone to blood through an acceleration of the linear rate of bone resorption. Alternately, the observed hypercalcemia could have resulted from increased intestinal calcium absorption since this change is known to occur

during phosphorus depletion (18). A decreased rate of mineralization was probably not the cause of the hypercalcemia because this change was found only in severely hypophosphatemic animals.

In addition to the finding that hypophosphatemia increases bone resorption, our results (Fig. 4) suggest

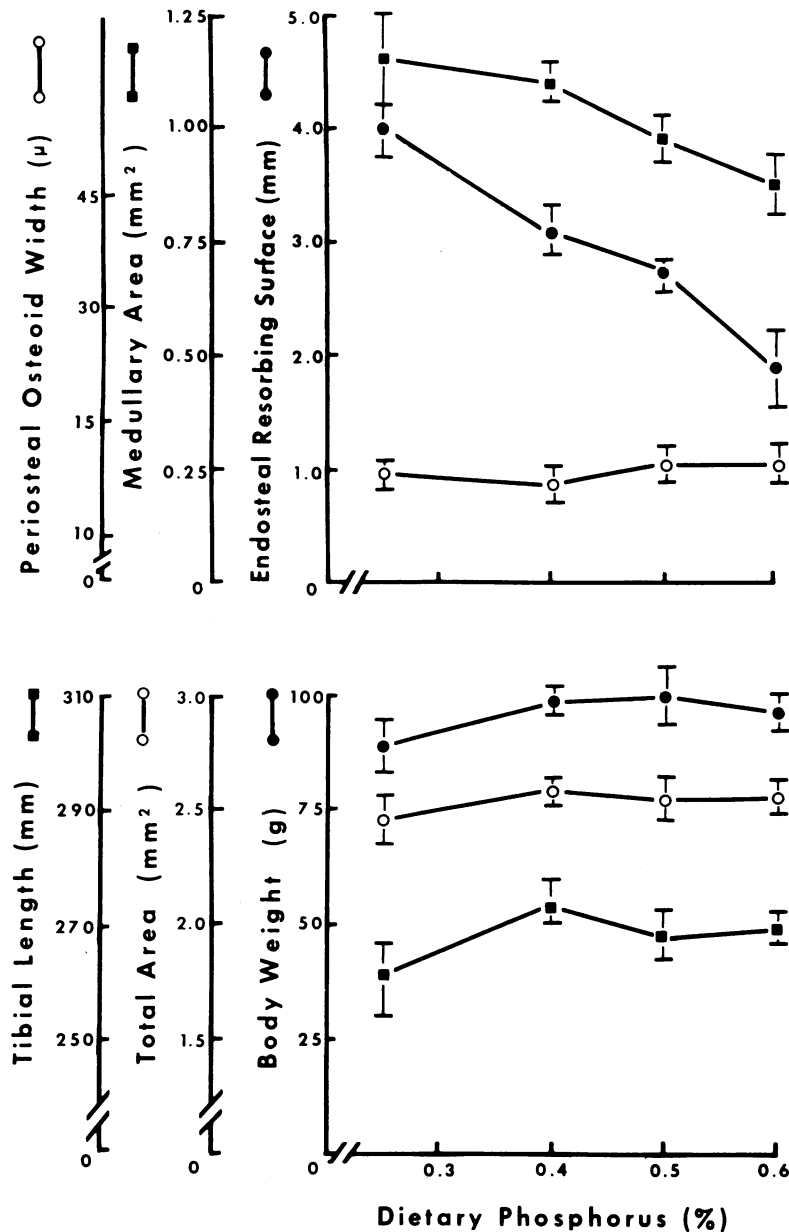


FIGURE 4 Effect of different dietary levels of phosphorus on medullary area and endosteal resorbing surface as compared with osteoid width and three parameters of growth (tibial length, body weight, and total area [i.e. the area encompassed by the periosteal perimeter of bone which includes both bone and medullary area]) in TPTX rats. Five to six rats were used for each dietary level of phosphorus, and each point represents the mean \pm SE.

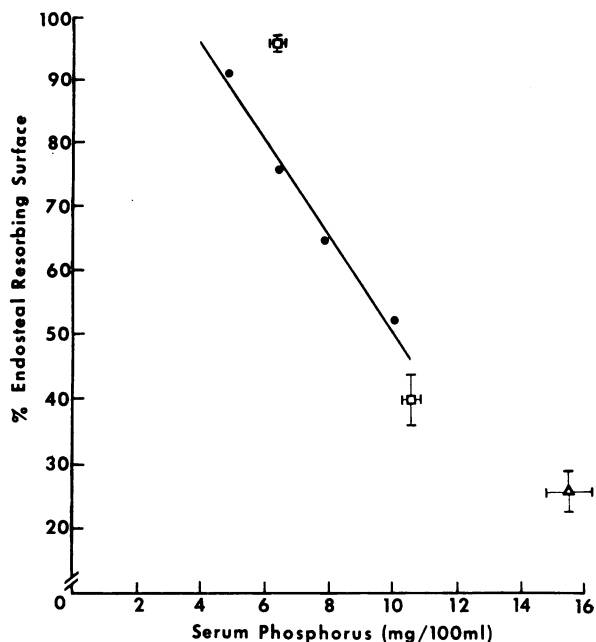


FIGURE 5 Relationship between serum phosphorus and per cent endosteal resorbing surface. The closed circles represent mean values obtained from groups of TPTX rats, five to six rats per group, fed different levels of dietary phosphorus. The line drawn through these four points was determined from the method of least squares. From 10 to 5 mg/100 ml of serum phosphorus, there is a linear relationship between per cent endosteal resorbing surface and serum phosphorus ($r = -0.99$, $P < 0.001$). The other points in this figure were obtained in separate experiments and therefore were not included in this correlation. The squares represent mean \pm SE from groups of intact rats, 12 rats per group. The triangle represents the mean \pm SE obtained from 12 TPTX rats which were fed a 1.2% calcium and a 0.6% phosphorus diet and which were slightly older than those represented by the closed circle.

that hyperphosphatemia decreases bone resorption, at least in TPTX animals. This observation is consistent with the results obtained in vitro by Raisz and Niemann (6). Studies in rats have been interpreted to indicate that increased calcium deposition in bone probably entirely accounts for the hypocalcemia occurring during a phosphate infusion (8). However, in view of our results and those of Raisz and Niemann (6), the possibility remains that the hypocalcemia during a phosphate infusion results in part from an inhibition of bone resorption.

In this study we showed that vitamin D deficiency reduced the effect of hypophosphatemia to increase the endosteal resorbing surface (Fig. 7), and in a previous study we showed that vitamin D deficiency also reduced the effect of endogenous parathyroid hormone to increase the endosteal resorbing surface (7). Thus, two quite different factors, parathyroid hormone and hypophosphatemia, both stimulate bone resorption optimally only when

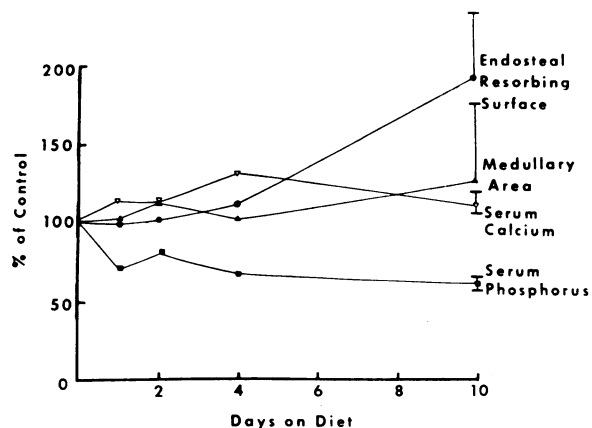


FIGURE 6 Effect of the length of time of feeding a low phosphorus diet (0.3%) on serum phosphorus and calcium, endosteal resorbing surface, and medullary area in TPTX rats. Each point is the mean or the mean \pm SE of six rats.

vitamin D is present. In addition, it has been shown that pharmacologic doses of vitamin D in vivo (19) and an active metabolite of vitamin D (25-hydroxycholecalciferol) in vitro (20) stimulate bone resorption. Thus, there is increasing evidence that vitamin D, or more probably one of its active metabolites, plays a fundamental role in the process of osteoclastic bone resorption.

In our experiments in intact rats, hypophosphatemia overcame the action of calcitonin to prevent hypercal-

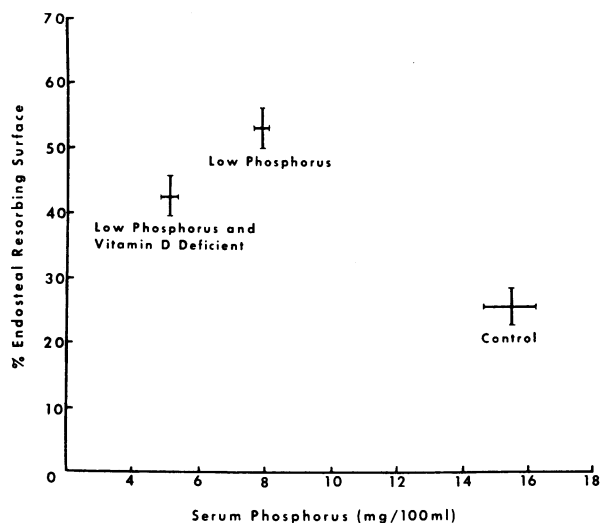


FIGURE 7 Relationship between changes in serum phosphorus and per cent endosteal resorbing surface in TPTX rats fed a low phosphorus (0.25%) and a vitamin D-deficient diet as compared with two other groups of TPTX rats: one fed a low phosphorus diet (0.25%) and one fed a normal phosphorus diet (0.55%). Each point represents the mean \pm SE of 10-15 rats.

cemia. Since a major effect of calcitonin is to decrease bone resorption (10), the findings of increased bone resorption and hypercalcemia in intact hypophosphatemic rats suggest that calcitonin does not effectively inhibit the increased bone resorption caused by hypophosphatemia. This interpretation is consistent with the findings of Copp and Kuczerpa who found that the hypocalcemic effect of calcitonin was substantially less in animals fed a low phosphorus diet than in those fed a normal or high phosphorus diet (21).

It has been shown that during phosphorus depletion, phosphorus is removed from bone and utilized in soft tissues, and only late in the course of phosphorus depletion is there a decrease in phosphorus concentration in soft tissues (1). This suggests that the stimulation of bone resorption in response to hypophosphatemia is a fundamental part of a homeostatic mechanism to maintain extracellular and probably intracellular phosphorus concentration at the expense of bone phosphorus. This concept is supported by our findings that the resorptive response to changes in serum phosphorus were not only marked but evident over a wide range of serum phosphorus levels including the normal range. This concept is also consistent with the hypothesis advanced by Tarlo (22) and others that bone originated not primarily as a supporting structure but as a store for phosphorus.

The marked inhibition of mineralization observed in this study supports the conclusion by de Deuxchaisnes and Krane that hypophosphatemia per se can account for the mineralization defect seen in patients with phosphate diabetes (23). In contrast with the results of this study, Kelly, Jowsey, Riggs, and Elveback found, in bone biopsies from patients with osteoporosis, a positive rather than a negative correlation between resorbing surface and serum phosphorus (24). However, since our results clearly establish that serum phosphorus is an important regulatory factor in bone resorption in experimental animals, we would also expect to see this relationship in human subjects. What remains to be clarified in human subjects is whether changes in serum phosphorus within the physiological range produce changes in bone resorption of sufficient magnitude to be of clinical importance.

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REFERENCES

1. Day, H. G., and E. V. McCollum. 1939. Mineral metabolism, growth, and symptomatology of rats on a diet extremely deficient in phosphorus. *J. Biol. Chem.* **130**: 269.
2. Freeman, S., and F. C. McLean. 1941. Experimental rickets—blood and tissue changes in puppies receiving a diet very low in phosphorus, with and without vitamin D. *Arch. Pathol.* **32**: 387.
3. Bloom, W. L., and D. Flinchum. 1960. Osteomalacia with pseudofractures caused by the ingestion of aluminum hydroxide. *J. Amer. Med. Ass.* **174**: 1327.
4. Copp, D. H., and A. P. Suiker. 1962. Study of calcium kinetics in calcium- and phosphorus-deficient rats with the aid of radiocalcium. In *Radioisotopes and Bone*. P. LaCroix and A. M. Budy, editors. F. A. Davis Co., Philadelphia. 1.
5. Raisz, L. G. 1970. The pharmacology of bone. *Fed. Proc.* **29**: 1176.
6. Raisz, L. G., and I. Niemann. 1969. Effect of phosphate, calcium and magnesium on bone resorption and hormonal responses in tissue culture. *Endocrinology.* **85**: 446.
7. Baylink, D., M. Stauffer, J. Wergedal, and C. Rich. 1970. Formation, mineralization, and resorption of bone in vitamin D-deficient rats. *J. Clin. Invest.* **49**: 1122.
8. Rasmussen, H., J. Feinblatt, N. Nagata, and M. Pechet. 1970. Effect of ions upon bone cell function. *Fed. Proc.* **29**: 1190.
9. Baylink, D., J. Wergedal, M. Stauffer, and C. Rich. 1970. Effects of fluoride on bone formation, mineralization and resorption in the rat. In *Fluoride in Medicine*. T. L. Vischer, editor. Hans Huber, Bern. 37.
10. Baylink, D., E. Morey, and C. Rich. 1969. Effect of calcitonin on the rates of bone formation and resorption in the rat. *Endocrinology.* **84**: 261.
11. Baylink, D., J. Wergedal, M. Stauffer, and C. Rich. 1970. Relationship between acid polysaccharide concentration and the initiation of bone mineralization in vivo. *Clin. Res.* **18**: 450. (Abstr.)
12. Wergedal, J. 1969. Enzymes of protein and phosphate catabolism in rat bone. I. Enzyme properties in normal rats. *Calcified Tissue Res.* **3**: 55.
13. Moore, E. W. 1970. Ionized calcium in normal serum, ultrafiltrates, and whole blood determined by ion-exchange electrodes. *J. Clin. Invest.* **49**: 318.
14. Stauffer, M., D. Baylink, J. Wergedal, and C. Rich. 1969. Defective bone mineralization in calcium-deficient rats. *Clin. Res.* **17**: 293. (Abstr.)
15. Wergedal, J. E., and D. J. Baylink. 1971. Factors affecting bone enzymatic activity in vitamin D-deficient rats. *Amer. J. Physiol.* **220**: 406.
16. Nichols, G., Jr., B. Flanagan, and J. v.d. Sluys Veer. 1969. Distortions of bone cell metabolism in uremia and their cause. *Arch. Intern. Med.* **124**: 530.
17. Rixon, R. H. 1968. Mitotic activity in the bone marrow of rats and its relation to the level of plasma calcium. *Curr. Mod. Biol.* **2**: 68.
18. Lotz, M., E. Zisman, and F. C. Bartter. 1968. Evidence for a phosphorus-depletion syndrome in man. *N. Engl. J. Med.* **278**: 409.

19. McLean, F. C. 1941. Activated sterols in the treatment of parathyroid insufficiency; review. *J. Amer. Med. Ass.* **117**: 609.
20. Trummel, C. L., L. G. Raisz, J. W. Blunt, and H. F. DeLuca. 1969. 25-Hydroxycholecalciferol: stimulation of bone resorption in tissue culture. *Science (Washington)*. **163**: 1450.
21. Copp, D., and A. Kuczerpa. 1966. Effect of age and dietary calcium and phosphorus on the response of TCT in the rat. *Proc. Can. Fed. Biol. Soc.* **9**: 54.
22. Tarlo, L. B. H. 1964. The origin of bone. In *Bone and Tooth*. H. J. J. Blackwood, editor. The Macmillan Company, New York. 3.
23. de Deuxchaisnes, C. N., and S. M. Krane. 1967. The treatment of adult phosphate diabetes and Fanconi syndrome with neutral sodium phosphate. *Amer. J. Med.* **43**: 508.
24. Kelly, P. J., J. Jowsey, B. L. Riggs, and L. R. Elveback. 1967. Relationship between serum phosphate concentration and bone resorption in osteoporosis. *J. Lab. Clin. Med.* **69**: 110.