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Effects of fasting, feeding, and bisphosphonate administration on serum calcitriol levels in phosphate-deprived rats

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Background. In a recent study, we showed in phosphate-deprived rats that morning feeding decreased serum phosphate and increased serum calcium values as compared with similar rats fasted overnight, and high doses of bisphosphonates did not reduce the magnitude of hypercalcemia. In the present study, we evaluated in phosphate-deprived rats whether serum calcitriol values were: (*1*) affected by the differences in serum phosphate induced by morning feeding and overnight fasting, (*2*) correlated with changes in serum phosphate levels, and (*3*) influenced by bisphosphonate administration.

Methods. Four groups of rats were studied: (*1*) low-phosphate diet (LPD; $P < 0.05\%$), (*2*) LPD + the bisphosphonate pamidronate (APD), (*3*) normal diet (ND; P 0.6%), and (*4*) ND + APD. Both diets contained 0.6% calcium. In rats receiving APD, high doses (0.8 mg/kg) were given subcutaneously four times during the study. On day 11, rats were sacrificed after an overnight fast or two to four hours after morning feeding.

Results. In the fed phosphate-deprived rats (LPD and LPD + APD), serum phosphate levels were less ($P < 0.05$) and serum calcium levels were greater ($P < 0.05$) than in similar rats fasted overnight. In rats on the ND (ND and ND + APD), no differences were observed between fed and fasted rats. In phosphate-deprived rats, serum calcitriol levels were greater (LPD, $P < 0.05$) or tended to be greater (LPD + APD, $P = 0.10$) in the fed than in the fasted groups. In APD-treated rats, serum calcitriol values were greater than in rats not given APD whether rats were (*1*) fed or fasted, or (*2*) on an LPD or ND. An inverse correlation was present between serum phosphate and serum calcitriol ($r = -0.58$, $P = 0.001$). In a stepwise regression model in which serum calcitriol was the dependent variable and independent variables were APD administration and serum calcium, phosphate, and PTH, serum phosphate ($P = 0.003$) had an inverse and APD ($P < 0.001$) administration a direct effect on serum calcitriol ($r^2 = 0.59$).

Conclusion. Calcitriol synthesis is rapidly inducible in rats during chronic phosphate deprivation, and the increase in serum calcitriol values is best attributed to feeding-induced decreases in serum phosphate. APD administration independently increases serum calcitriol levels in rats on normal and phosphate-deprived diets. Finally, whether our results in the rat are applicable to the clinical setting should be evaluated because in previous human studies of dietary phosphate restriction, serum calcitriol measurements were performed the morning after an overnight fast.

Previous studies have shown that dietary phosphate deprivation in animals and dietary phosphate restriction in humans stimulate calcitriol production [1–6]. It has also been shown that both hypophysectomy and diabetes reduce the stimulation of calcitriol by phosphate deprivation [7–10], and treatment with either insulin, growth hormone, or insulin-like growth factor-I (IGF-I) restores the capacity of phosphate deprivation to stimulate calcitriol [8, 10–12]. In the aforementioned studies, the effect that several days of phosphate deprivation had on calcitriol stimulation was evaluated, but the effect that feeding and fasting had on serum calcium, phosphate, and calcitriol levels was not specifically addressed. In previous studies in phosphate-deprived rats, feeding after an overnight fast has been shown to induce rapidly marked hypophosphatemia and hypercalcemia [13–15]. In studies of phosphate restriction in young healthy men, often there has not been any difference in serum phosphate values obtained after an overnight fast between subjects on normal diets and those on phosphate-restricted diets [16–19]. In studies in young healthy women, a small difference in serum phosphate was present after an overnight fast between volunteers on a normal diet and those on a phosphate-restricted diet [16, 17]. Finally, it has been shown that the small difference present in fasting serum phosphate values becomes much greater after breakfast because of the postfeeding decrease in serum phosphate values in those on the phosphate-restricted diet [20].

In previous studies, the capacity of dietary phosphate deprivation to increase serum calcitriol values has been determined primarily in the fasted state or the effect of fasting and feeding was not specifically studied. Because

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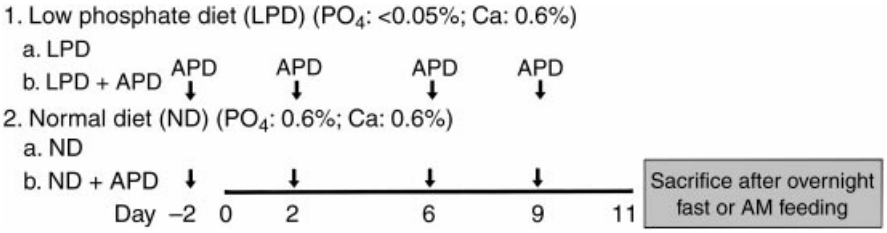

Fig. 1. Schematic diagram of the study design. Shown are the four groups of rats, the diets used in the study, the days on which Pamidronate (APD) was administered, and the day of sacrifice.

feeding rapidly changes serum phosphate and calcium values in the phosphate-deprived rat, it would seem reasonable to evaluate the effect that feeding has on the simultaneous values of serum calcitriol and phosphate in such rats. In a recent publication, we showed that two to four hours of feeding in rats with prolonged dietary phosphate deprivation rapidly decreased serum phosphate and increased serum calcium values [15]. We also showed that these postfeeding changes were not prevented with bisphosphonate treatment and that bisphosphonate treatment increased serum calcitriol values. In the present report, we show that (*1*) in phosphate-deprived rats, feeding rapidly increases serum calcitriol levels and the decrease in serum phosphate induced by feeding correlates with the magnitude of increase in serum calcitriol levels, and (*2*) bisphosphonate administration independently increases serum calcitriol values in rats whether maintained on a normal or low-phosphate diet.

METHODS

In a recent publication [15], we evaluated in phosphate-deprived rats the effect of bisphosphonate (Pamidronate, Novartis, Summit, NJ, USA) administration on the development of hypercalcemia and the calcemic response to parathyroid hormone (PTH). In that study, approximately one half of the rats were sacrificed on day 11 of the study diet, and in the other rats, an Alzet pump containing 1-34 rat PTH was implanted for two days to evaluate the calcemic response to PTH. In the rats sacrificed on day 11, blood was obtained by aortic puncture, and in the other half, blood was obtained on day 11 from the tail vein before implantation of the Alzet pump. Only in the rats in which blood was obtained from the aorta was the blood volume sufficient to measure calcitriol. Even in a few of these latter rats, the amount of serum available was found to be insufficient to measure calcitriol by the independent laboratory (Nichols Institute, San Juan Capistrano, CA, USA) to which the samples were sent.

Male Wistar rats weighing 160 to 180 g were used for the study. During procedures, rats were anesthetized with intraperitoneally administered ketamine 7.5 mg/100 g (Ketaset; Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine 0.5 mg/100 g (AnaSed; Lloyd Laboratories, Shenandoah, IA, USA). Rats were housed in individual cages, given 14 g of food daily between 8 and 9 a.m., and were allowed free access to water.

The two diets used during the study were provided by the same supplier (ICN, Cleveland, OH, USA) and contained 0.6% calcium and 100 IU of vitamin D per 100 g of diet. Dietary phosphate content was either <0.05% (low phosphate diet; LPD) or 0.6% (normal diet; ND). As shown in Figure 1, two groups received the LPD, and two groups received the ND. In one LPD and one ND group, the bisphosphonate, Pamidronate (APD), was administered subcutaneously two days before the start of the study diet and on days 2, 6, and 9 during the 11 days of the study diet. The dose of APD used (0.8 mg/kg) is a high dose [21, 22], which, as we recently showed, results in a marked alteration of osteoclast morphology and a reduction in the osteoblast surface [15]. Thus, the four groups of rats studied were (*1*) LPD, (*2*) LPD + APD, (*3*) ND, and (*4*) ND + APD. Blood was obtained on day 11 after an overnight fast (approximately 12 to 14 hours without food) or two to four hours after early morning feeding.

Serum calcium and phosphate were measured with specific kits (Sigma, St. Louis, MO, USA), serum creatinine with a creatinine analyzer (Beckman, Fullerton, CA, USA), serum PTH with an immunoradiometric assay specific for intact rat PTH (Nichols, San Juan Capistrano, CA, USA), and serum calcitriol with a radioreceptor assay by Nichols Institute.

Statistics

Comparisons of the data among the eight groups were assessed by one-way analysis of variance (ANOVA) followed by a post hoc test, the Fisher LSD, for multiple comparisons. For the correlation between two variables, the Pearson's correlation was used. For these tests, a P value < 0.05 was considered significant. A stepwise regression was used to determine the effect of independent variables on a dependent variable. Results are shown as the mean ± SE.

RESULTS

There were no differences among the eight groups in serum creatinine values (ANOVA, $P = 0.49$), the weight

Table 1. Serum calcium, phosphate, parathyroid hormone (PTH), and calcitriol values in rats sacrificed by aortic puncture on Day 11

	Calcium[a]	Phosphate[a]	PTH[a]	Calcitriol[a]
Serum	*mg/dL*		*pg/mL*	
Fasted				
LPD ($N = 4$)	10.58 ± 0.28	9.36 ± 0.89	16 ± 6	235 ± 42
LPD + APD ($N = 4$)	11.36 ± 0.78	7.12 ± 0.96	15 ± 8	366 ± 29[e]
ND ($N = 4$)	9.53 ± 0.02	8.70 ± 0.61	85 ± 14	198 ± 8
ND + APD ($N = 4$)[b]	9.52 ± 0.17	8.76 ± 0.87	92 ± 25	303 ± 18
Fed				
LPD ($N = 4$)	12.42 ± 0.45[c]	5.00 ± 0.58[c]	6 ± 1	347 ± 58[c]
LPD + APD ($N = 3$)	12.93 ± 0.37[c]	4.63 ± 0.64[c]	7 ± 1	460 ± 49[d,e]
ND ($N = 4$)	9.60 ± 0.24	8.67 ± 0.12	61 ± 17	198 ± 13
ND + APD ($N = 4$)[b]	10.03 ± 0.18	9.80 ± 0.91	74 ± 7	387 ± 51[f]
ANOVA	<0.001	<0.001	<0.001	<0.001

Data are mean ± SE.
[a]Comparisons not shown between groups on normal diet and groups on low phosphate diet
[b]For calcitriol, the $N = 2$
[c]$P < 0.05$ vs. same group, fed vs. fasted
[d]$P = 0.10$ vs. same group, fed vs. fasted
[e]$P < 0.05$ vs. LPD in fed and fasted grouping
[f]$P < 0.05$ vs. ND in fed grouping

at sacrifice (ANOVA, $P = 0.29$), and the weight gained during the study (ANOVA, $P = 0.13$). Table 1 shows the serum calcium, phosphate, PTH, and calcitriol values in fasted and fed rats. The values for serum calcium, phosphate, and PTH are similar to those in our recent publication that included additional rats in which blood was obtained from the tail vein [15]. As shown in Table 1, serum calcium values were greater ($P < 0.05$) and serum phosphate values were less ($P < 0.05$) in fed than in fasted phosphate-deprived rats (LPD and LPD + APD). In rats on the ND (ND and ND + APD), there was no difference in serum calcium and phosphate values between the fed and fasted groups. PTH values were less in the groups on the LPD (LPD and LPD + APD) than in the groups on the ND (ND and ND + APD), but there were no differences in PTH values between fed and fasted rats in the same dietary group. Serum calcitriol values were greater ($P < 0.05$) in the fed than in the fasted phosphate-deprived group (LPD) and also tended to be greater ($P = 0.10$) in the fed than in the fasted LPD + APD group. The administration of APD increased serum calcitriol levels in rats both on a phosphate-deprived and ND, independent of whether rats were fed or fasted.

As shown in Figure 2, when a comparison between serum phosphate and calcitriol was performed, an inverse correlation was present ($r = -0.58$, $P = 0.001$). Separating the rats into fed and fasted groups (Fig. 3) improved the correlation ($r = -0.67$, $P = 0.01$) between serum phosphate and calcitriol in fed rats (Fig. 3A), but reduced the correlation ($r = -0.30$, $P =$ NS) in fasted rats (Fig. 3B). Moreover, APD treatment tended to increase serum calcitriol levels across a wide range of serum phosphate values (Fig. 3C).

Stepwise regression was performed to determine the effect of independent variables (phosphate, calcium, PTH, and bisphosphonate treatment) on the dependent variable, serum calcitriol (Table 2). While serum phosphate, calcium, and PTH were continuous variables, bis-

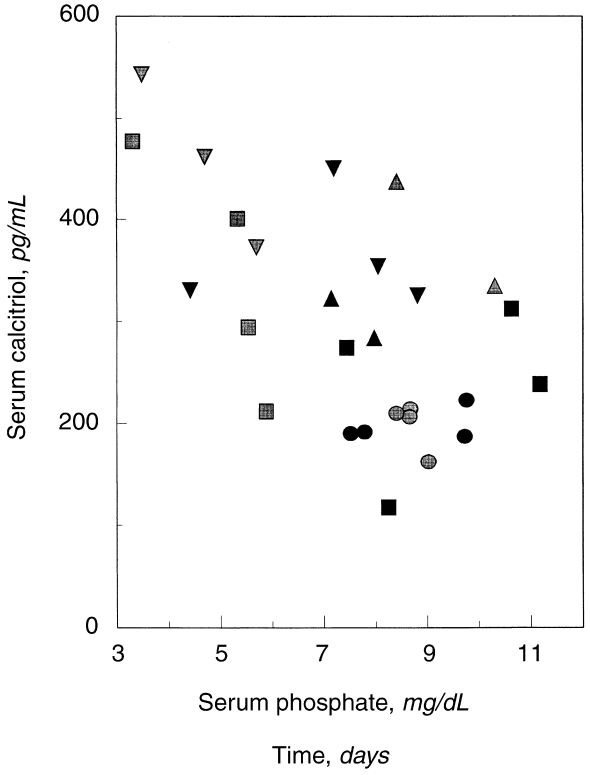

Fig. 2. Correlation between serum calcitriol and serum phosphate in rats on a normal diet (ND) or a low protein diet (LPD). The correlation between serum calcitriol and phosphate is shown for all rats sacrificed on day 11 ($r = -0.58$; $P = 0.001$). Symbols are: (■) LPD-fasted; (▩) LPD-fed; (▼) LPD + APD-fasted; (▽) LPD + APD-fed; (●) ND-fasted; (○) ND-fed; (▲) ND + APD-fasted; (△) ND + APD-fed.

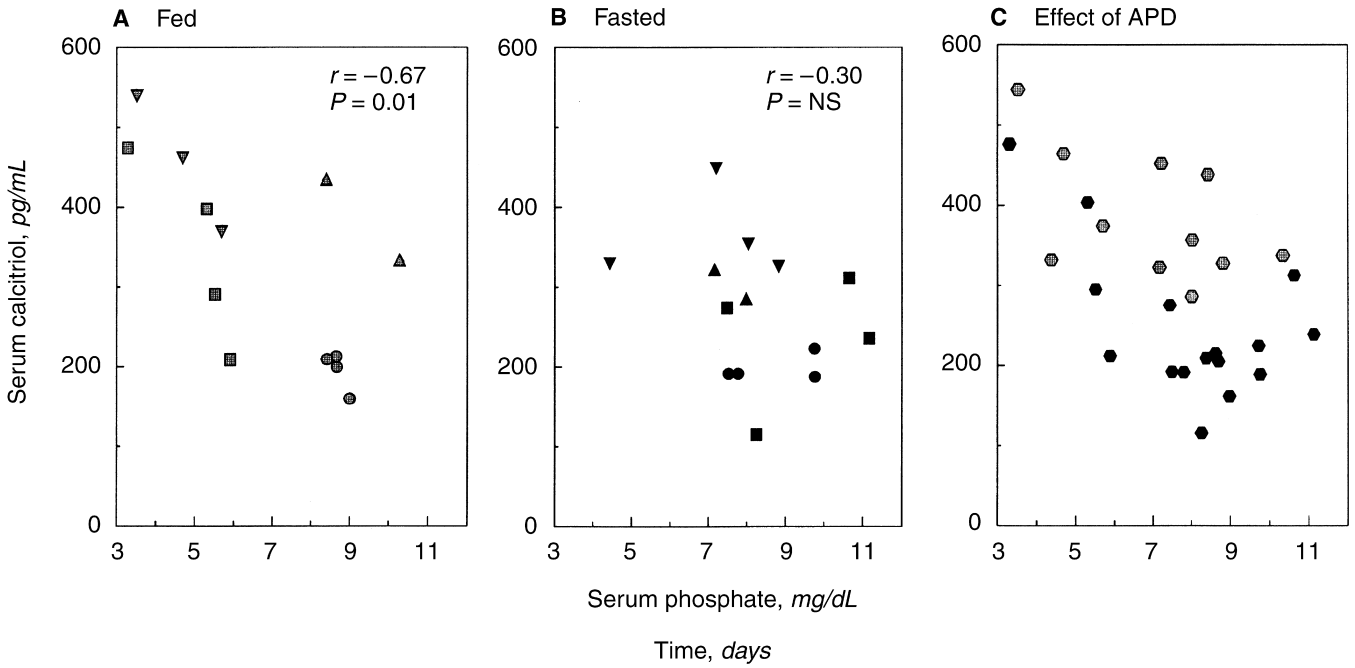

Fig. 3. Correlation between serum calcitriol and serum phosphate and the effect of (*A*) feeding, (*B*) fasting, and (*C*) APD treatment. Symbols in A (fed) and B (fasted) are: (■) LPD; (▼) LPD + APD; (●) ND; (▲) ND + APD. Symbols in C are: (●) no APD; (○) APD. In the latter group, the correlation between serum calcitriol and phosphate in rats treated with APD was $r = -0.54$, $P = 0.08$, and in rats not treated with APD, the correlation was $r = -0.60$, $P = 0.01$.

Table 2. Stepwise regression for rats in which serum calcitriol values were available

	t value	*P* value
All rats		
Dependent variable		
Serum calcitriol		
Independent Variables		
Serum phosphate	−3.4	0.003
Bisphosphonate administration	3.8	<0.001
Serum calcium	0.8	NS
PTH	0.8	NS
	$r^2 = 0.59$	
Fed rats		
Dependent variable		
Serum calcitriol		
Independent variables		
Serum phosphate	−4.1	0.003
Bisphosphonate administration	4.7	0.002
Serum calcium	−1.9	0.10
PTH	0.7	NS
	$r^2 = 0.85$	
Fasted rats		
Dependent variable		
Serum calcitriol		
Independent variables		
Serum phosphate	0.9	NS
Bisphosphonate administration	3.9	0.002
Serum calcium	0.7	NS
PTH	−1.2	0.27
	$r^2 = 0.63$	

phosphonate treatment was either absent or present. As shown in Table 2, when all rats ($N = 27$) were included, the r^2 value was 0.59; serum phosphate had a strong inverse effect, and bisphosphonate administration had a strong positive effect on serum calcitriol values. The effects of calcium and PTH were not significant. When only fed rats ($N = 13$) were evaluated, the r^2 value was 0.85. Again, serum phosphate had a strong inverse effect, and bisphosphonate administration had a strong positive effect on serum calcitriol values (Table 2). Serum calcium also had a minor effect. In fasted rats ($N = 14$), the r^2 value was 0.63. Bisphosphonate administration continued to have a strong positive effect, and PTH had a minor effect; however, serum phosphate no longer had a significant effect (Table 2).

DISCUSSION

In rats maintained for 10 days on the LPD, morning feeding after an overnight fast induced a rapid decrease in serum phosphate values and a rapid increase in serum calcium values. Associated with the rapid decrease in serum phosphate values was a similarly rapid increase in serum calcitriol values. In rats maintained on the ND, morning feeding did not change serum phosphate, calcium, and calcitriol values. Finally, in rats maintained on either the ND or LPD and in both the fed and fasted states, bisphosphonate administration independently increased serum calcitriol values.

In previous studies in rats maintained on phosphate-deprived or restricted diets, feeding has been shown to produce a rapid decrease in serum phosphate values [13, 14]. As a result of elegant studies performed in rats on phosphate-restricted (0.2% P) diets, it was suggested that (*1*) fasting increased serum phosphate values because it resulted in an efflux of phosphate into the extracellular space [14], and (*2*) calcitriol treatment together with an increase in serum calcium in thyroparathyroidectomized rats enhanced the net exit of phosphate from the extracellular space into soft tissue or bone [23]. While the rats in the present study were not parathyroidectomized, PTH values in the rats on the LPD were very low. Thus, in our phosphate-deprived rats, it is possible that the feeding-induced increases in serum calcium and calcitriol levels were important in facilitating the shift of phosphate into cells.

In previous studies in animals and humans, the increase in serum calcitriol values during phosphate deprivation has been shown to be due to an increase in calcitriol production [3, 24–26] and not to a decrease in the metabolic clearance rate [25, 26]. The rapid increase in serum calcitriol values that we observed within four hours after the start of feeding was somewhat surprising. Moreover, the strong inverse correlation that was observed between serum calcitriol and the degree of hypophosphatemia, and the significance of the serum phosphate concentration in the stepwise regression suggests that the feeding-induced decrease in serum phosphate was an important factor in the rapid increase of serum calcitriol levels.

The results of the present study also pose some potentially important questions. In essentially all previous clinical studies of dietary phosphate restriction, blood for calcitriol was drawn in the morning after an overnight fast [16–20, 25, 27]. Thus, it is possible that the true magnitude of calcitriol stimulation was not observed. Because the degree of dietary phosphate restriction is much less in clinical studies than in studies in rats, it could be argued that feeding might not induce the same degree of hypophosphatemia and calcitriol stimulation. However, in the study in rats by Trohler, Bonjour, and Fleisch, the effect of fasting and feeding on serum phosphate was observed with only a moderately phosphate-restricted diet (0.2% P) [14]. Moreover, in the clinical studies of Portale, Halloran, and Morris, normal men on a phosphate-restricted diet had a much greater decrease in serum phosphate after breakfast than when ingesting a normal or high-phosphate diet [19, 20]. As far as we can determine, little information is available on feeding-induced changes in serum calcitriol values in such situations. While studies in normal humans during normal dietary calcium and phosphate ingestion have shown diurnal variations in serum calcitriol levels to be small [28–30], the effect of dietary phosphate restriction was not evaluated in these studies.

Our determination of the effect of feeding and fasting on serum calcium, phosphate, PTH, and calcitriol levels was performed in rats that had been on the LPD for 10 days. Other studies in the rat have shown that a similar LPD for even shorter durations results in decreases in serum phosphate values. It has been shown that by 24 hours of the LPD, serum phosphate values decrease [4, 31] and serum calcitriol levels increase [4]. Others have shown that serum phosphate values decrease by four to six hours after the start of the LPD [32, 33]. Perhaps even more interesting is that Levine et al have shown that in rats previously maintained on the ND, gavage with the LPD resulted in a decrease in serum phosphate values by one hour [34]. It is not known whether these rapid changes in serum phosphate are associated with increases in serum calcitriol.

In previous studies in rats and humans on phosphate-deprived or restricted diets, hypophysectomy or growth hormone deficiency has been shown to blunt or prevent the stimulation of calcitriol [7, 9, 10, 35]. Other studies have suggested that IGF-I is an important mediator of calcitriol stimulation during phosphate restriction [9–12, 36, 37]. However, previous studies have reported that IGF-I levels do not increase rapidly after feeding or other stimuli [38, 39]. Finally, insulin has also been suggested to be an important mediator of the calcitriol response to phosphate deprivation [8, 10]. In the present study, the effect of feeding on changes in phosphate and calcitriol stimulation was rapid, and the role—if any—of growth hormone, insulin, or even IGF-I remains to be determined.

The present study also shows that bisphosphonate administration independently increases serum calcitriol values. Studies from the 1970s showed that the first-generation bisphosphonate, etidronate, inhibited 1-α-hydroxylase activity [40, 41]. In subsequent studies in which third-generation bisphosphonates, which contain a basic primary nitrogen atom on the R_2 alkyl side chain, were used for the treatment of hypercalcemia of malignancy [42], Paget's disease [42–44], and juvenile osteoporosis [45] or were given to normal volunteers [46], an elevation of serum calcitriol levels was observed. In these studies, the elevation in serum calcitriol levels was attributed to concomitant increases in PTH levels. However, several studies have reported that the administration of third-generation bisphosphonates to parathyroidectomized rats [47, 48] or to cyclosporine-treated rats in which PTH levels did not change [49] resulted in increases in serum calcitriol values. In one of these studies, it was also reported that the in vitro addition of bisphosphonates increased 1-α-hydroxylase activity in mitochondria obtained from kidneys removed from bisphosphonate-treated rats [48]. In our study, bisphosphonate administration had an effect on calcitriol stimulation that by stepwise regression was shown to be essentially equal to that of phosphate deprivation. The effect of bisphosphonate administration was

observed both in rats on NDs and LPDs. Furthermore, the increase in serum calcitriol levels was even observed in phosphate-deprived rats with very low PTH levels.

The present study contained only a relatively small number of rats in each group because, besides our intention to evaluate the effect of bisphosphonates on serum calcium and phosphate levels in phosphate-deprived rats, the original study was designed to evaluate the calcemic response to a PTH infusion [15]. Since the measurement of serum calcitriol levels was not part of the original design, serum calcitriol values could not be measured in rats in which blood samples were obtained from the tail vein. However, the findings that significant differences were present among the groups, an inverse correlation was observed between serum calcitriol and phosphate, and the strong independent effects shown for both phosphate and bisphosphonate during the stepwise regression analysis suggest that the observed differences were real.

In summary, (*1*) in chronically phosphate-deprived rats, feeding after an overnight fast rapidly increased serum calcium and calcitriol values and decreased serum phosphate values. (*2*) The increase in serum calcitriol values correlated with feeding-induced decreases in the serum phosphate level. (*3*) In rats on an ND, feeding had no effect on serum calcitriol and phosphate values. (*4*) High-dose bisphosphonate administration independently increased serum calcitriol values in rats on LPDs and NDs; this effect was observed in both the fed and fasted states. In conclusion, calcitriol synthesis appears to be rapidly inducible in rats during chronic phosphate deprivation, and the increase in serum calcitriol values is best attributed to feeding-induced decreases in serum phosphate. Finally, whether our results of feeding-induced calcitriol stimulation in phosphate-deprived rats are applicable to the clinical setting should be evaluated, because in previous human studies of dietary phosphate restriction, measurements of serum calcitriol were performed the morning after an overnight fast.

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