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Regulation of Serum 1α,25-Dihydroxyvitamin D_3 by Calcium and Phosphate in the Rat

Abstract. *A new radioreceptor assay was used to quantify changes in serum concentration of 1α,25-dihydroxyvitamin D_3 in rats with low calcium or low phosphate diets. Low availability of either ion elicits a fivefold increase in the circulating concentration of 1α,25-dihydroxyvitamin D_3. The enhancement of 1α,25-dihydroxyvitamin D_3 concentration in response to calcium deficiency is dependent on the presence of the parathyroid or thyroid glands (or both), suggesting that this effect is mediated by parathyroid hormone. In contrast, the response to phosphate deficiency is independent of these glands and may result from an action of low serum phosphate concentration or some factor associated with phosphate depletion on the renal synthesis of the 1α,25-dihydroxyvitamin D_3 hormone.*

Vitamin D_3 is metabolized to 25-hydroxyvitamin D_3 (25-OHD_3) (*1*) and subsequently to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] (*2*). The latter conversion occurs exclusively in the kidney (*3*) and produces what is considered to be the hormonal form of the vitamin. In stimulating both intestinal calcium transport and bone calcium mobilization, 1α,25-$(OH)_2D_3$ is the most active and the fastest acting metabolite of vitamin D (*4*).

The production of this hormone is influenced by the calcium and vitamin D status of the animal (*5, 6*). The primary regulated step appears to be the 1α-hydroxylation of 25-OHD_3 in the kidney (*6*); however, the mechanism and details of this endocrine regulation are poorly understood. DeLuca and his co-workers, on the basis of the in vivo conversion of radioactive 25-OHD_3 to 1α,25-$(OH)_2D_3$, have reported (*5*) that hypocalcemia in the rat stimulates the production of 1α,25-$(OH)_2D_3$, and that this stimulation is dependent on the presence of the parathyroid glands (*7*). In contrast to these findings, and also to those of Fraser and Kodicek (*8*), MacIntyre and associates have observed that in both rats (in vivo) (*9*) and chicks (in vitro) (*10*) parathyroid glands are not essential for the production of 1α,25-$(OH)_2D_3$. Moreover, Tanaka and DeLuca (*11*) have reported that phosphate deficiency also results in enhanced production of the 1α,25-$(OH)_2D_3$ hormone. To date, all experiments investigating the control of 1α,25-$(OH)_2D_3$ formation have used either the measurement of the renal 1α-hydroxylase in vitro, or the ability of the animal to metabolize 25-OHD_3 to dihydroxy derivatives. Since these methods measure the conversion of 25-OHD_3 to 1α,25-$(OH)_2D_3$ at any given time and are affected by the pool size of 25-OHD_3 precursor, they may not reflect the steady state concentration of this hormonal product in the blood. In any case, the regulation of the renal synthesis and secretion of 1α,25-$(OH)_2D_3$ appears to be a complex endocrine feedback system which will probably require the results of a number of experimental approaches before it can be adequately described.

Since the recent development of a specific and sensitive radioreceptor assay for 1α,25-$(OH)_2D_3$ (*12*), it has become possible to eliminate the effects of alterations in hormone degradation rates and determine the total serum activity of the hormone. Using this assay, it is possible to directly assess the effects of proposed regulators of 1α,25-$(OH)_2D_3$ serum concentration. The data obtained from the measurement of the circulating concentration of this hormone, combined with the data obtained by in vitro enzyme activity studies and in vivo conversion experiments, should clarify the control of 1α,25-$(OH)_2D_3$ formation as well as the relationships between vitamin D and mineral homeostasis.

In our study, the serum 1α,25-$(OH)_2D_3$ concentration was measured in response to changes in dietary calcium and phosphorus content and in response to surgical removal of the thyroid and parathyroid (TPTX) glands of rats that were subsequently given replacement thyroxine. Thus, the TPTX rats were deficient in calcitonin and parathyroid hormone. A total of 169 male weanling Holtzman rats, either intact or TPTX, were fed one of the following partially synthetic diets: (i) a normal diet (0.6 percent calcium, 0.6 percent phosphorus); (ii) a low calcium diet (0.01 percent calcium, 0.6 percent phosphorus); (iii) a low phosphorus diet (0.6 percent calcium, 0.04 percent phosphorus); or (iv) high calcium diet (1.8 percent calcium, 0.65 percent phosphorus, 20 percent lactose). The concentration of salts (potassium phosphate, calcium carbonate, and potassium chloride) were varied in these diets in order to achieve the desired levels of calcium and phosphorus; fibrin was used as the source of protein because of its low phosphorus content; and when lactose was used, it was substituted for sucrose. The constituents of these different diets (*13*) were essentially the same except for inorganic salt content. All diets contained vitamin D_3 (2 international units per gram of diet). After 2 weeks on the diets, blood was obtained from the rats by cardiac puncture for measurement of serum calcium, phosphorus, and 1α,25-$(OH)_2D_3$. The serum sample (*14*) for the 1α,25-$(OH)_2D_3$ assay was extracted with a mixture of methanol and chloroform (2 : 1, by volume), and the vitamin D hormone was isolated by successive Sephadex LH-20 and Celite column chromatography (*15*). A sensitive, competitive binding assay with a high affinity, saturable receptor system from the hormone's target tissue was used to measure the 1α,25-$(OH)_2D_3$ concentration in the serum (*12*). This technique has been shown to be a reliable and valid method for detecting hormone concentrations as low as 1.0 ng per 100 ml of serum (*12*), and recent modifications (*15, 16*) have improved the speed of this radioreceptor assay.

In intact animals (no surgery) on a normal calcium and phosphate diet, circulating 1α,25-$(OH)_2D_3$ was 17.3 ng/100 ml (Table 1). A marked increase of this hormone to 90 or 82 ng/100 ml occurs when the dietary intake of either calcium or

phosphate, respectively, is limited in intact animals. However, the mechanism for this increase is distinctly different in calcium-deficient and phosphate-deficient intact animals. As with the intact rat, TPTX animals fed the phosphate-deficient diet had marked elevation of serum 1α,25-$(OH)_2D_3$ (91.3 ng/100 ml), which indicates that neither parathyroid hormone nor calcitonin is necessary for the increase in serum 1α,25-$(OH)_2D_3$ in response to phosphate deprivation (Table 1). The possibility that hypophosphatemia or some other factor closely associated with serum phosphate concentration is responsible for the increase in 1α,25-$(OH)_2D_3$ is supported by the work of Tanaka and DeLuca (*11*), who have shown that phosphate deprivation causes an increase in the conversion of 25-OHD_3 to 1α,25-$(OH)_2D_3$. In contrast, TPTX calcium-deficient animals, although hypocalcemic, did not exhibit the dramatic increase in serum 1α,25-$(OH)_2D_3$ seen in intact calcium-deficient animals. Since parathyroid gland volume is significantly increased in such calcium-deficient, intact animals (*17*), it is possible that the increase in serum 1α,25-$(OH)_2D_3$ in these animals is a result of high parathyroid hormone (PTH) levels. Also, normal circulating concentrations of PTH appear to be required to maintain an adequate serum level of 1α,25-$(OH)_2D_3$, since the 1α,25-$(OH)_2D_3$ concentration falls to 6.2 ng/100 ml in normocalcemic TPTX rats. These data are consistent with the concept that PTH functions as an important regulator of the circulating level of 1α,25-$(OH)_2D_3$ (*7*). Our findings do not exclude the possibility that hypocalcemia per se influences serum 1α,25-$(OH)_2D_3$ to some degree. For example, in TPTX animals, hypocalcemia is associated with a significantly higher serum 1α,25-$(OH)_2D_3$ than in normocalcemic TPTX animals (12.3 ng/100 ml and 6.2 ng/100 ml). Since intact calcium-deficient rats develop only a slight, but statistically significant, hypocalcemia (Table 1), it is probable that PTH corrects the serum calcium deficit by mobilizing bone mineral. And the fact that serum 1α,25-$(OH)_2D_3$ is strikingly increased in these slightly hypocalcemic animals suggests that PTH may be much more dominant than serum calcium levels as a regulator of 1α,25-$(OH)_2D_3$ concentration.

The dual control by calcium and phosphate in modulating the serum concentration of 1α,25-$(OH)_2D_3$ is consistent with the following concept of homeostatic regulation of these serum ions. Two signals, low serum calcium and low serum phosphate, are postulated as primary initiators of this hormonal system. Low circulating calcium acts, via an increase in PTH, to enhance the 1α,25-$(OH)_2D_3$ concentration in the serum. The sterol then acts on the bone and intestine to increase the circulating calcium and phosphate. Parathyroid hormone also acts on bone to mobilize calcium and phosphate and on kidney to produce phosphaturia. Since the enhanced renal excretion of phosphate counterbalances the augmented serum phosphate, the net result produced from the original hypocalcemic stimulus is a selective increase in serum calcium. Phosphate deprivation (probably mediated by hypophosphatemia) also acts to elevate serum 1α,25-$(OH)_2D_3$, which in turn increases gut absorption and bone liberation of both calcium and phosphate. The consequent elevation of serum calcium leads to a depression of PTH secretion, which, together with the hypercalcemia, results in an increase in urine calcium but a decrease in urine phosphate excretion. Accordingly, the net result of the initial hypophosphatemic stimulus is a selective increase in serum phosphate. Thus, in response to calcium or phosphate deficiency, the action of PTH on renal electrolyte excretion emerges as an important element in the dual control of serum calcium and phosphate. If this complex control mechanism for calcium and phosphate were also operative in humans, it would provide a means for environmental adaptations in normal individuals, and it might provide some insight into the mechanism of clinical disorders of calcium and phosphate metabolism, such as idiopathic hypercalciuria.

The radioreceptor assay for 1α,25-$(OH)_2D_3$ has made it possible, for the first time, to measure the circulating concentration of this hormone in experimental animals. Our data strengthen the conclusions of DeLuca and co-workers (*5, 7, 11*) that both hypocalcemia and hypophosphatemia augment the synthesis of 1α,25-$(OH)_2D_3$, and that this enhancement is achieved by different mechanisms. One significant question that remains is how these factors operate on the kidney cell to accelerate the formation of 1α,25-$(OH)_2D_3$. Some work by Rasmussen *et al.* (*18*) suggests that the 1α-hydroxylase of isolated renal tubules can be activated by PTH or its proposed intracellular mediator, cyclic adenosine monophosphate (cyclic AMP). However, reduction of phosphate concentration in renal homogenates or mitochondria does not activate the 1α-hydroxylase (*19*). Most of the reported effects on the 1α-hydroxylase enzyme require a number of hours of exposure of an animal to various challenges, indicating that induction-repression of the enzyme may be the molecular mode of its regulation (*5–11*). Since one of the classic effects of PTH on renal cells is to lower intracellular phosphate, it is possible that both PTH (cyclic AMP) and phosphate depletion enhance the 1α-hydroxylase through a common intracellular mechanism.

Table 1. Serum calcium, phosphorus, and 1α,25-$(OH)_2D_3$ in intact or TPTX rats fed normal, calcium-deficient, or phosphate-deficient diets.

Group	No. of sets	No. per set†	Dietary Ca (%)	Dietary P (%)	Serum* Calcium (mg/100 ml)	Serum* Phosphorus (mg/100 ml)	Serum* 1α,25-$(OH)_2D_3$ (ng/100 ml)
Normal; intact	4	7	0.6	0.6	11.4 ± 0.5	10.0 ± 1.5	17.3 ± 1.4
Calcium-deficient; intact	5	2	0.01	0.6	10.1 ± 0.8‡	9.9 ± 1.0	90.0 ± 3.8§
Phosphate-deficient; intact	4	2	0.6	0.04	13.0 ± 0.4‖	4.8 ± 0.3‖	82.3 ± 7.0
Normal; TPTX	7	10	1.8	0.65	11.1 ± 0.7	10.1 ± 1.1	6.2 ± 2.1¶
Calcium-deficient; TPTX	5	9	0.6	0.6	8.0 ± 0.2#	15.7 ± 2.7#	12.3 ± 3.4**
Phosphate-deficient; TPTX	4	2	0.6	0.04	13.4 ± 0.5#	5.4 ± 0.4#	91.3 ± 8.4§

*Value ± standard deviation. †Number of animals per set; that is, 28 normal, intact animals were studied. ‡Significantly different from normal-intact, $P < .05$. §Not significantly different from phosphate-deficient, intact $P > .05$. ‖Significantly different from normal-intact, $P < .005$. ¶Significantly different from normal-intact, $P < .005$. #Significantly different from normal-TPTX, $P < .005$. **Significantly different from normal-TPTX, $P < .01$; significantly different from normal-intact, $P < .025$.

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14. Serum samples from seven to ten rats were pooled for assay of $1\alpha,25\text{-}(OH)_2D_3$; 10- to 20-ml samples were usually used. In rats with enhanced circulating concentration of $1\alpha,25\text{-}(OH)_2D_3$, such as calcium-deficient intact, phosphate-deficient intact, and phosphate-deficient TPTX, only 3 to 4 ml of serum was required.
15. The method of extraction and chromatographic purification of $1\alpha,25\text{-}(OH)_2D_3$ described by P. F. Brumbaugh *et al.* [*Biochemistry* **13**, 4091 (1974)] was modified as follows. Silicic acid column chromatography was deleted from the isolation scheme. After adding [^{3}H]-$1\alpha,25(OH)_2D_3$ (6 c/mmole; 2000 count/min) and extraction of sterols, $1\alpha,25\text{-}(OH)_2D_3$ was purified by two successive columns of Sephadex LH-20 (1 by 15 cm; 5 g). Elution was performed with 65 percent chloroform in hexane and the hormone emerged between 40 and 95 ml elution volume. Final purification of the hormone was effected by Celite liquid-liquid partition chromatography [M. R. Haussler and H. Rasmussen, *J. Biol. Chem.* **247**, 2328 (1972)]. The solvent system was 10 percent ethyl acetate in hexane (mobile) and 45 percent water in ethanol (stationary). Micro-Celite columns (0.8 by 8.5 cm) were packed into 10-ml pipets fitted with glass wool plugs, and eluted with mobile phase and $1\alpha,25\text{-}(OH)_2D_3$ emerged when the elution volume was between 7 and 22 ml. The final sample was evaporated under nitrogen and solubilized in 400 μl of ethanol. After determination of yield by counting 50 μl of each sample for tritium (yields ranged from 50 to 75 percent), radioreceptor assays were performed on 100- μl portions.
16. The radioreceptor assay was modified slightly from P. F. Brumbaugh *et al.* [*Biochemistry* **13**, 4091 (1974)] as follows. Intestinal mucosa (6 g) from two rachitic chicks was homogenized in 25 ml of 0.25*M* sucrose in 0.05*M* tris-HCl (*p*H 7.4), 0.025*M* KCl, 0.005*M* $MgCl_2$. The cytosol fraction was obtained by centrifugation at 100,000*g* for 1 hour. Chromatin was prepared from crude nuclei (isolated from homogenate at 1,000*g* for 10 minutes) by homogenizing successively in one 25-ml portion of 0.8 m*M* EDTA and 25 m*M* NaCl, *p*H 8; one 25-ml portion of 1 percent Triton X-100 and 0.01*M* tris-HCl, *p*H 7.5; and one 25-ml portion of 0.01*M* tris-HCl, *p*H 7.5. The chromatin was harvested by sedimentation at 30,000*g* for 10 minutes after each washing. The entire chromatin pellet from 6 g of mucosa was reconstituted with the cytosol fraction by homogenization to create a cytosol-chromatin system for the competitive binding assay. The assay was carried out as described, except that 20 μl of distilled ethanol was added to each assay tube (containing nitrogen-dried sterol) just before the addition of 200 μl of reconstituted receptor system. The ethanol aided in solubilizing the sterols and reduced nonspecific binding. Triplicate assays were performed on all samples; the variation between assays was 10 to 15 percent.
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Organophosphorus and Methyl Carbamate Insecticide Teratogenesis: Diminished NAD in Chicken Embryos

Abstract. *Studies with 36 organophosphorus and 12 methyl carbamate compounds establish a correlation between reduction in nicotinamide adenine dinucleotide (NAD) levels and severity of teratogenic signs in chicken embryos, a relation supported by reversal of these effects by nicotinamide derivatives. Diminished NAD occurring at organophosphorus and methyl carbamate concentrations as low as 0.6 to 2.0 parts per million in the egg constitutes a newly recognized biochemical lesion induced by the two most important classes of insecticide chemicals.*

Organophosphorus (OP) and methyl carbamate (MC) inhibitors of acetylcholinesterase have gradually replaced the chlorinated insecticides so that they are now the insect control agents used most frequently and in the largest amounts. It is therefore important to define the nature and mechanism of any deleterious effect of OP and MC compounds not attributable to disruptions in the cholinergic system. This report concerns such an effect, the mechanism of teratogenesis in chicken embryos, and establishes that over various test conditions the severity of the teratogenic signs is related to the degree of reduction in the embryo nicotinamide adenine dinucleotide (NAD) level.

Many investigations (*1–7*) with chicken embryos establish that about 20 different OP compounds (including dicrotophos, Diazinon, malathion, and malaoxon) and several methyl carbamates (including eserine and carbaryl) produce similar or identical developmental defects (involving some or all of micromelia, abnormal beak, reduced body size, retarded down development, gross edema, and wryneck) when injected into the yolk sac at day 0 to 5 of incubation. No definite relation between structure and teratogenic activity has emerged from these studies; that is, dicrotophos (as the *cis*-crotonamide isomer) and eserine sulfate are teratogenic at 0.6 to 2.0 ppm in the egg, whereas closely related compounds such as tri-*o*-cresyl phosphate and the *trans*-crotonamide isomer of dicrotophos are not, even at 200 ppm. The teratogenic signs are almost completely alleviated, except for the malformation of the neck, when dicrotophos, Diazinon, malathion, malaoxon, eserine sulfate, and carbaryl are supplemented with nicotinamide or nicotinic acid; on a similar basis, the teratogenesis induced by dicrotophos is alleviated with any one of many precursors or derivatives of nicotinamide or nicotinic acid including the NAD cofactors (NAD, NADH, NADP, NADPH) (*1, 3–5*). These observations impose strict limits on any proposal for the biochemical mechanism involved in induction of this type of teratogenesis. Such proposals include (i) no involvement of acetylcholinesterase or the cholinergic system (*4, 5*); (ii) reduced uptake of endogenous tryptophan from the yolk (*5*); (iii) reduced sulfated mucopolysaccharide and RNA content and increased glycogen storage and calcification in the developing tibiotarsus (*6*); and (iv) phosphorylation or carbamoylation of a yolk sac membrane site initiating a sequence of events leading to embryonic abnormalities (*7*). However, not any one of these observations or hypotheses adequately explains all aspects of the phenomenon.

Several of the OP- and MC-teratogenic signs (micromelia, abnormal beak, reduced body size, and retardation of down development) resemble those produced by 6-aminonicotinamide (6-AN), whereas 3-acetylpyridine (3-AP) yields different embryonic abnormalities (muscular hypoplasia, most noticeable in the legs) (*8*). Both 6-AN and 3-AP are nicotinamide antagonists that are stated to act, at least in part, by diminishing the NAD levels in chicken embryos (*8, 9*), chicken limb mesodermal cell cultures (*10*), and mammals (*11*). Studies with embryonic chicken limbs and cultures of their mesodermal cells indicate the importance of embryo NAD levels in the control of muscle and cartilage development in that myogenic cells are observed when NAD levels are high and chondrogenic cells are seen when NAD levels are low (*12*).

These interrelations suggest the possibility that the OP and MC teratogens may act in part by diminishing the embryo NAD. This new hypothesis was subjected to a series of tests, correlating the embryo NAD at day 12 with the severity of teratogenic signs at day 19, after injection of teratogens on day 4 of incubation.

Fertile, white Leghorn eggs incubated at 37.5°C and 73 percent relative humidity were treated under sterile techniques by injection into the yolk sac with a methoxytriglycol solution (30 μl) of an OP or MC compound (*13*) in the presence or absence of a candidate-alleviating agent. Embryo NAD was assayed spectrophotometrically against a standard curve for authentic NAD after addition of yeast alcohol dehydrogenase and ethanol to form reduced NAD (NADH) (*14*). Other eggs from the same treated group were used to observe and record the teratogenic signs, rated