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Effects of Dietary Vitamin D Levels on the *in Vitro* Mineralization of Chick Metaphyses[1, 2] (38132)

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The failure of rachitic osteoid to mineralize *in vivo* has been ascribed to the undersaturation of the serum with respect to bone mineral. This conclusion is based primarily on experiments that have shown that rachitic hypertrophic cartilage calcifies *in vitro* when exposed to normal serum or inorganic solutions approximating normal serum electrolyte levels (1–3).

Lamm and Neuman (4) were able to qualitatively demonstrate *in vitro* mineralization of rachitic and normal bone. However, a quantitative measurement of mineralization has proven difficult because demineralized bone does not readily induce crystal formation from solutions having normal calcium and phosphate concentrations (5).

The primary purpose of the present study was to determine if vitamin D and normal serum calcium levels are necessary for the formation of bone matrix that is competent for mineral deposition. Undemineralized metaphyseal bone was used to determine if there was any quantitative differences in the mineral uptake *in vitro* by bone from vitamin D-deficient chicks, normal chicks and chicks fed a high, but non-toxic, level of vitamin D. An additional purpose of this study was to determine to what extent an elevated dietary level of calcium could mimic vitamin D with respect to the amount of mineral deposited *in vivo* and the uptake of mineral *in vitro*.

Materials and Methods. Animals. One-day-old Hi-Line White Leghorn cockerels were housed in a windowless room and fed a rachi-

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TABLE I. Vitamin D_3 and Calcium Additions to the Rachitogenic Diet.

Group	Dietary regimen	Additions/g of diet	
		Vitamin D_3 (I.U.)[a]	Ca (mg)[b]
I	— D, Control Ca	0	0
II	Control D, Control Ca	1.4	0
III	High D, Control Ca	70.0	0
IV	— D, Control Ca	0	0
V	— D, High Ca	0	14
VI	Control D, Control Ca	1.4	0
VII	Control D, High Ca	1.4	14

[a] The vitamin D_3 (Sigma Chemical Co., crystalline cholecalciferol) was dissolved in corn oil. The diets to which no vitamin D_3 was added contained an equal volume of corn oil.

[b] Calcium above the control level (14 mg/g diet) was added as $CaCO_3$.

togenic diet[3] containing 1.4% (w/w) Ca, 1.1% P and 0.25% Mg *ad libitum*. One series of chicks (Groups I, II and III) was fed this diet with various amounts of vitamin D_3 added. Later, a second series (Groups IV, V, VI and VII) was fed the diet with various amounts of vitamin D_3 and calcium added. These additions to the diet are shown in Table I. At the end of 2 weeks (all Groups) and 4 weeks (Groups I, II and III), six chicks from each group were sacrificed by decapitation.

Serum Ca and bone ash. At the time of sacrifice, a blood sample was collected for serum Ca determination by atomic absorption spectrophotometry (6). The left tibia (Groups I, II and III) and the left femur (Groups IV, V, VI and VII) were removed and freed of adhering tissues, extracted first with ethanol and then with diethyl ether (7) in a Soxhlet extraction apparatus to obtain dry weight. After the solvents were removed under vacuum, the fat-free bones were ashed for 24 hr at 650°.

Incubations. The incubation media were prepared from the following three stock solutions: (a) 0.0107 *M* KH_2PO_4, 0.117 *M* KCl, 0.01 *M* barbituric acid; (b) 0.0056 *M* $CaCl_2$, 0.138 *M* KCl, 0.01 *M* barbituric acid; (c) 0.155 *M* KCl, 0.01 *M* barbituric acid. The pH of each solution was adjusted to 7.4 with KOH, and each solution had an ionic strength of 0.165 (9). Neomycin sulfate (100 mg/liter) was added to the media to minimize bacterial growth (10). Media were prepared immediately before use such that the molar Ca/P was 1.67, and the calcium–phosphate product was either 0.5, 1.0, 1.5 or 2.0 $(mM)^2$.

The right tibia was removed from each chick at the time of sacrifice, and freed of adhering tissue. The periosteum was stripped away, and the epiphyseal cartilage was scraped from the bone (8). The proximal-most 1 mm of the metaphysis of each tibia was cut off, placed in an individual vessel containing ice-cold isotonic KCl, and cut radially into fragments which were blotted on filter paper and weighed immediately before they were used in the incubations. Fragments not used for the incubations were freeze-dried to constant weight. Half of these fragments from each tibia were ashed at 650° to constant weight; the total nitrogen content of the other half was determined (11).

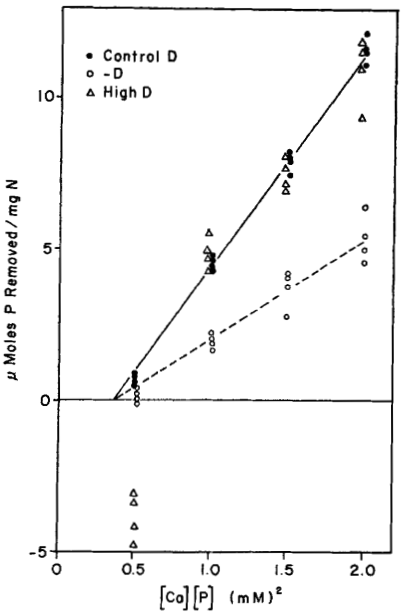

FIG. 1. The effects of dietary levels of vitamin D on the *in vivo* uptake of phosphate by metaphyseal slices from two-week-old chicks. The chicks were fed the rachitogenic diet without supplements (—D), supplemented with 1.4 I.U. D_3/g (control D) or with 70 I.U. D_3/g (high D). All of the animals in this first experimental series were fed control levels of Ca (14 mg/g diet). The individual data are plotted, and the regression lines are drawn for the control D and — D metaphyseal slices.

Four metaphyseal fragments of tibia from each of 4 animals from Groups I, II and III and from each of 3 animals from Groups IV, V, VI and VII were used in the incubations. One fragment from each tibia was placed in an individual vial containing 10 ml of medium at each concentration product. The fragments were selected, on the basis of the results of a preliminary experiment, to have approximately the same ash (1.0–1.5 mg), regardless of dietary regimen. Duplicate vials containing 10 ml of medium at each concentration product but without bone served as blank controls. All of the vials were incubated for 5 days at 37° with constant agitation. At the end of the incubation, the contents of each vial were filtered with a tared 0.45 μm Millipore filter.

[3] Diet No. 170650, General Biochemicals, Inc., Chagrin Falls, Ohio.

TABLE II. Serum Calcium and Whole Bone Ash.

Group	Dietary Regimen	Serum Ca[a] (mg/100 ml) 2 weeks	Serum Ca[a] (mg/100 ml) 4 weeks	Bone Ash[a] % of Fat-free Dry Weight 2 weeks	Bone Ash[a] % of Fat-free Dry Weight 4 weeks
I	— D, Control Ca	8.4 ± .28[b]	6.2 ± .20[b]	37.4 ± .60[b]	32.6 ± .53[b]
II	Control D, Control Ca	10.4 ± .28	10.3 ± .20	43.4 ± .60	43.9 ± .53
III	High D, Control Ca	10.0 ± .28[c]	10.4 ± .20[c]	43.3 ± .60[c]	43.6 ± .53[c]
IV	— D, Control Ca	8.0 ± .16[d]		36.2 ± .53[d]	
V	— D, High Ca	8.9 ± .16[d, e]		41.3 ± .53[e, f]	
VI	Control D, Control Ca	10.6 ± .16		42.0 ± .53	
VII	Control D, High Ca	10.6 ± .18[f]		43.4 ± .53[f]	

[a] Mean ± standard error of 5 or 6 animals.
[b] Significantly different ($P < .001$) from Group II.
[c] Not significantly different ($P > .20$) from Group II.
[d] Significantly different ($P < .001$) from Group VI.
[e] Significantly different ($P < .01$) from Group IV.
[f] Not significantly different ($P > .20$) from Group VI.

Inorganic phosphate (12) and calcium (6) were determined in the filtrate.

Bacterial contamination was not evident since no more than a total of 5 colonies per flask grew on blood agar plates which were streaked with a loop of the medium from each of the vials prior to filtration.

Statistics. Data from each group of chicks were subjected to analysis of variance. Regression lines were calculated by the least squares method. Standard errors (SE) were calculated from the residual error of the appropriate analysis of variance. Significance of differences between mean values and regression coefficients were evaluated using a one-tailed Student's *t*-test.

Results. Evidence of rickets in chicks fed the −D, control calcium diet (Groups I and IV, see Table II) consisted of reduced serum calcium concentrations and reduced bone ash percentages compared to chicks fed the control D, control Ca diet (Groups II and VI, respectively). Chicks fed the high D, control Ca diet (Group III) had serum Ca and bone ash values that were not different from those fed the control D, control Ca diet (Group II).

Hypocalcemia was evident in the chicks on the −D, high Ca regimen (Group V), but the hypocalcemia was not as severe as in the

TABLE III. Ash Content of the Metaphyseal Slices.

Group	Dietary regimen	Ash as % of dry weight[a] 2 weeks	Ash as % of dry weight[a] 4 weeks
I	— D, Control Ca	30.9 ± 1.26[b]	27.6 ± 1.12[b]
II	Control D, Control Ca	45.5 ± 1.38	46.4 ± 1.12
III	High D, Control Ca	54.3 ± 1.26[c]	52.8 ± 1.12[c]
IV	— D, Control Ca	30.2 ± 1.38[d]	
V	— D, High Ca	47.5 ± 1.38[e, f]	
VI	Control D, Control Ca	47.1 ± 1.38	
VII	Control D, High Ca	45.8 ± 1.51[f]	

[a] Mean ± standard error 5 or 6 samples.
[b] Significantly different ($P < .001$) from Group II.
[c] Significantly different ($P < .01$) from Group II.
[d] Significantly different ($P < .001$) from Group VI.
[e] Significantly different ($P < .001$) from Group IV.
[f] Not significantly different ($P > .2$) from Group VI.

chicks on the −D, control Ca regimen (Group IV). Even though Group V was hypocalcemic, the ash content of the bones from these chicks was not significantly different from that of the chicks on the control D, control Ca diet (Group VI). The elevated dietary calcium had no effect on the serum Ca or bone ash of the animals receiving the control D diet (Group VII).

The relative differences in the ash contents of the metaphyseal slices (Table III) between the respective Groups was the same as for the intact bone (Table II), with one exception: the slices from the high D animals (Group III) had a higher ash content than those from the controls (Group II).

The metaphyseal fragments from the two-week-old Group II chicks (control D, control Ca) took up additional mineral at each calcium–phosphate concentration product (Fig. 1). The uptake by Group I (−D, control Ca) fragments at each calcium–phosphate concentration product, except 0.5 $(mM)^2$, was significantly less ($P < .001$) than the uptake by the Group II fragments. The respective linear regression coefficients, calculated from the two sets of data, were also significantly different ($P < .001$). The y-intercepts of the respective regression lines were the same for the Group I and Group II fragments, indicating that the *in vivo* deposited mineral in the fragments had the same apparent equilibrium calcium–phosphate product, 0.36 $(mM)^2$.

The fragments from the two-week-old Group III chicks (high D, control Ca) lost mineral to the medium which had the lowest calcium–phosphate concentration product (0.05 $(mM)^2$). The average amount of mineral lost to this medium was 94% of the excess mineral in the metaphyseal slices from the Group III chicks (Table II). In the higher calcium–phosphate products, the Group III fragments took up amounts of mineral that were not different from the Group II fragments.

There was no difference ($P > .4$) between the respective uptake of mineral by metaphyseal fragments from the Group I, II and III chicks at two weeks (Fig. 1) compared to those at 4 weeks of age. This relationship was true for the respective means at each concentration product, the linear regression coefficients, and the y-intercepts of the regression lines.

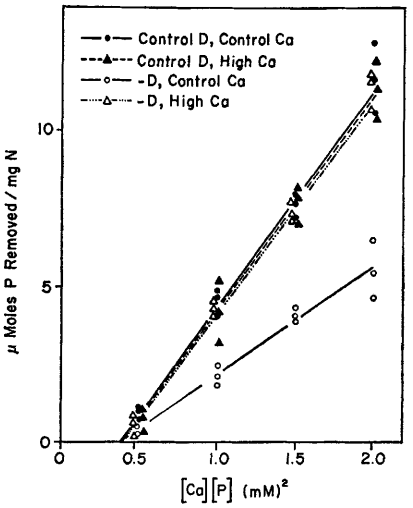

FIG. 2. The effects of dietary levels of vitamin D and Ca on the *in vitro* uptake of phosphate by metaphyseal slices from two-week-old chicks. The chicks were fed the rachitogenic diet (−D, control Ca); or this diet supplemented with either 35 mg $CaCO_3$/g (−D, high Ca), or 1.4 I.U. D_3/g (control D, control Ca) or 1.4 I.U. D_3/g and 35mg $CaCO_3$/g (con-D, high Ca). The individual data are plotted, and the regression line is drawn for each dietary group.

In the second experimental series (Groups IV, V, VI and VII), there were no significant differences ($P > .5$) in mineral uptake by the metaphyseal fragments during the incubations between Groups V (−D, high Ca), VI (control D, control Ca) and VII (control D, high Ca) whether the comparisons were made between the means at each calcium–phosphate product or between the linear regression coefficients. The metaphyseal fragments from the chicks of group IV (−D, control Ca) was significantly less ($P < .001$) than the uptake by Group V (−D, high Ca) or by Group VI (control D, control Ca) and all calcium–phosphate products, except 0.5 $(mM)^2$.

The data obtained from the incubation studies were reproducible between the 2 experimental series. The respective linear regression coefficients of Groups I and IV and of Groups II and V were not significantly different ($P > .5$).

Discussion. The hypocalcemia and reduced bone ash of the chicks fed the −D, control calcium diet (Groups I and IV) are similar to the results reported by many other investigators. Previous reports from this (13) and

other laboratories (14) have shown that chicks fed a rachitogenic diet develop hypocalcemia at 1 week and a reduced bone ash at 2 weeks.

Doubling the calcium content of the rachitogenic diet increased the serum calcium (Group V), although these chicks were still hypocalcemic compared to those fed vitamin D (Group VI). However, they were able to deposit a normal amount of bone mineral. This observation is in agreement with the earlier observations of Migicovsky and Emslie (15) who found that the bone ash of chicks fed a rachitogenic diet increased with increasing dietary levels of calcium. However, Au and Raisz (16) reported that the chronic injection of calcium chloride into vitamin D-deficient rats elevated the serum calcium but had no effect on bone ash. These differences could be assigned to a species difference, a difference in the severity of rickets (measured by the reduction in bone ash), or the acidosis resulting from the injection of calcium as the chloride.

The chicks fed the high-calcium diet containing vitamin D (Group VII) were normocalcemic and had a normal bone mineral content. This result is similar to those reported earlier for rats (17) and for chickens (15).

The percentage ash of the entire tibias of the chicks fed the high D diet (Group III) was not different from that of those fed the control D diet (Group II). The high D animals were also normocalcemic. However, the ash content of the metaphyseal slices from these animals indicated that they were hypermineralized.

The differences between the analyses of the metaphyseal bone and whole bone at 2 and at 4 weeks, reported here, and between 1 week and older whole bone, reported earlier (13), could be explained by the replacement of hypermineralized trabecular bone by normally mineralized cortical bone as the bone grows. In this way, the hypermineralization of the trabecular bone would be masked, since the proportion of trabecular to cortical bone decreases as the bone length increases.

The *in vitro* incubation experiments showed that the extra mineral present in the metaphyses from the high-D chicks is very labile (Fig. 1) at a calcium–phosphate concentration product at which the control D and −D bone took up little or no mineral from the medium. The remaining mineral appeared to behave identically to that in the control D fragments.

The metaphyseal fragments from the rachitic chicks (Groups I and IV) were less efficient in removing mineral from the incubation media than were those from the control-D animals (Groups II and VI). This difference was found between fragments at 2 and at 4 weeks of age. The high calcium diet eliminated this difference.

Lamm and Neumann (4) reported that demineralized sections of bone from rachitic and vitamin D-fed rats attained the same degree of mineralization, as judged by the von Kossa technique, when they were exposed to solutions having a calcium–phosphate product of 3.5 $(mM)^2$ or greater. The quantitative results reported here show that bone from rachitic chicks takes up mineral *in vitro,* but that the amount of mineral uptake was less than that for vitamin D-fed chicks.

The difficulties encountered in the remineralization of demineralized bone have been recognized. Reactivity of the ϵ-amino groups of lysine and hydroxylysine of bone collagen with fluorodinitrobenzene is decreased if this reagent were added after demineralization, as compared to the reactivity if the reagent were present during decalcification (18, 19). Solomons and Neuman (5) cited this as evidence for a steric or chemical change in the bone collagen during decalcification. The change could explain why demineralized bone does not recalcify well.

A mechanism to explain the lesser degree of calcification of rachitic osteoid *in vitro* can be postulated. The matrix is formed and secreted by the osteoblasts, and it is rendered competent as a calcification matrix. However, this competency is short-lived; the matrix loses its ability to be calcified through some chemical or steric change if the appropriate amount of mineral is not present during this period.

The data support the concept that hypomineralization of rachitic bone is mainly due to undersaturation of the serum (an indirect effect of vitamin D) rather than a basic defect in the mineralizing matrix since the hypomineralization can be corrected by dietary calcium as well as by vitamin D. At the same

time, however, the hypermineralization of bone in normocalcemic chicks fed the high D-diet suggests that vitamin D can have an effect on bone that is not mediated by changes in serum calcium levels.

Summary. Chicks fed a rachitogenic diet became hypocalcemic and formed hypomineralized bones compared with chicks fed a control diet. Undemineralized metaphyseal slices from rachitic chicks incubated for five days in media with four different Ca-P concentration products had significantly less mineral deposited than slices from control chicks or chicks fed the rachitogenic diet supplemented with Ca. The latter chicks formed normally mineralized bones and had serum Ca values intermediate between those of the rachitic and control chicks. The data support the concept that hypomineralization of rachitic bone *in vivo* is mainly due to reduced serum Ca levels, but in addition show that hypomineralized rachitic bone, when tested *in vitro,* has lost some of its ability to serve as a calcification matrix.

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