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Skeletal Response to Exogenous Ascorbic Acid by Vitamins D_3 Deficient Chicks.* (31019)

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Possible metabolic relationships in bone tissue between ascorbic acid and vitamin D have not been an area of extensive research interest. Despite the paucity of information regarding such possibilities some recent work suggests that the two vitamins may have certain affiliations. Skeletal uptake and release of Ca^{45} was markedly enhanced in vit. D_3 deficient chicks which were progeny of ascorbic acid-fed forebears(1). The addition of this water soluble vitamin to rachitogenic diets(2) also indicated an alteration in other skeletal responses. Principal changes included influences on energy metabolism and mineral release during *in vitro* incubation of the bone tissue. Evidence of a highly correlated association between skeletal energy utilization and demineralization was also exhibited by animals given ascorbic acid(2). The present study concerns the skeletal response to ascorbic acid which was administered subcutaneously in advanced stages of vit. D_3 deficiency.

Methods and materials. One-day-old Leghorn chicks were placed on 2 dietary treatments consisting of a complete diet(3) or this same ration without supplementary vit. D_3. The animals were given feed and water free-choice throughout the experiment. They were reared in electrically-heated batteries in a room with incandescent lights. After 26 days of dietary treatment, the vit. D_3 deficient group was divided into 2 groups of 10 animals each. These animals and 10 others which had received the complete diet were injected subcutaneously for 2 consecutive days with solutions as shown in Table I. Ascorbic acid was administered in 0.9% saline solution at a level of 5 mg per 100 g body weight per day. The concentration of this vitamin in the saline solution was controlled so that no individual received more than 1.5 ml per day and all were given a comparable volume per unit of body weight.

Twenty-four hours following the last injection the animals were sacrificed, the right tibia removed immediately, cleaned of adhering soft tissue and placed in cold 0.9% saline solution. After cooling, the bone was split lengthwise and portions of the compact bone, free of marrow, were harvested and placed in 2.8 ml of cold media (pH 7.4) containing the following in μmoles: Tris (hydroxymethyl) aninomethane 20, $MgSO_4$ 15, glucose 10, NaCl 155 and ADP (adenosine diphosphate) 10. Hexokinase was added at 0.5 mg per flask. Incubation was conducted in the Dubnoff metabolic shaker at 37°C for 3 hours using air as the gas phase.

Chemical determinations of the media included glucose(4), lactic acid(5), hydroxyproline(6), calcium(7), phosphate(8). Noncollagenous nitrogen (NCN)(9) was determined on the incubated bone samples. Similar bone tissue from the tibia was fat-extracted for 16 hours with pentane, dried to a constant weight at 105°C, ashed overnight

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TABLE I. *In Vivo* Response.

Diet	Injection	NCN μg/100 mg bone	% bone ash
Control	Saline	668 ± 45*	65.0 ± .6
Vit D_3 deficient	"	735 ± 49	60.8 ± .6‡
" "	Saline + ascorbic acid	829 ± 57	55.0 ± 1.2‡

* Mean ± SE of mean.
† Significantly different from controls at the 1% confidence level.
‡ Significantly different from the vit D_3 deficient group at the 1% confidence level.

at 600°C, weighed and per cent ash calculated.

Results. The chicks given the complete diet were included to illustrate the adequacy of the vit. D_3 deficiency diet for effecting rachitic symptoms. It is evident that the individuals given the deficient diet were inhibited in skeletal development as exhibited by the highly significant reduction in per cent bone ash (Table I). This observation and the elevation of NCN are similar to results previously reported(2). The *in vitro* results (Table II) are also similar to those observed in that study.

An *in vivo* response by vit. D_3 deficient animals to the injected ascorbic acid was exhibited by the reduction in bone ash (Table I). This observation is particularly interesting since the vit. D_3 deficiency alone had initially caused a highly significant reduction in the per cent bone ash. The elevation of NCN for the ascorbate group (Table I) is also evidence for an *in vivo* alteration although this difference was not significant from the deficient group.

In vitro changes were consistently observed between the 2 deficiency groups (Table II). Significant alterations were evident in the ascorbic acid group for all measurements except glucose utilization. In general, the results indicated a decreased rate of cellular activity accompanied by a lower rate of mineral release in vit. D_3 deficient individuals given the ascorbic acid.

Discussion. The marked reduction in per cent bone ash in vit. D_3 deficient chicks given exogenous ascorbic acid could be explained on a basis of an enhanced resorptive activity. No such effect has been attributed to ascorbic acid, although an increased turnover of skeletal Ca^{45} has been noted in its presence(2). In this study, the significantly reduced release of calcium and phosphate to the media does not support the notion that active resorptive processes were associated with the reduced bone ash, although the increased hydroxyproline content does. Normally, it is believed that the bone resorptive process is a simultaneous destruction of the entire bone, therefore the data concerning mineral and hydroxyproline release are paradoxical for the group given ascorbic acid.

An alternative explanation could also be given concerning the influence on bone matrix. If matrix formation were sufficiently stimulated by the ascorbic acid injection, it seems probable that the reduced bone ash could be attributed to a change in ratio between organic and inorganic bone fractions. Evidence has been presented implicating ascorbic acid in hydroxyproline formation(10-12). In one case(12), the effect was observed *in vitro* and occurred after 6 hours. This effect and the rapidity in which the reaction can occur gives credence to the idea that the reduction

TABLE II. *In Vitro* Observations.

Diet	Injection	(μg/mg NCN/hr) Glucose uptake	(μg/mg NCN/hr) Lactic acid recovery	Released by bone to media (μg/100 mg bone/hr) Calcium	Phosphate	Hydroxyproline
Control	Saline	895 ± 79	90 ± 9	106 ± 4	126 ± 2	32 ± 5
Vit D_3 deficient	"	637 ± 69*	71 ± 5	102 ± 4	68 ± 2†	34 ± 3
" "	Saline + ascorbic acid	531 ± 98	52 ± 8‡	81 ± 6§	29 ± 3§	47 ± 3§

* † These symbols represent a comparison between control and vit D_3 deficient groups only and indicate confidence levels of 5 and 1% respectively.
‡ § These compare the 2 deficient groups only and indicate similar confidence levels as * and †.

in per cent ash was due to a stimulated production of organic matrix. If this were correct, it could then be suggested that vit. D_3 deficiency in the chick inhibited either the synthesis or physiological activity of ascorbic acid.

Summary. Subcutaneous injection of ascorbic acid given to vit. D_3 deficient chicks resulted in a highly significant reduction in per cent bone ash suggesting that the introduction of this compound had stimulated bone resorption. Calcium and phosphate release by incubating bone tissue did not support this idea, although the movement of hydroxyproline did. An alternative explanation is based on the possibility that the marked change in per cent bone ash resulted from a change in the organic-inorganic ratio of bone tissue. If this were true, it would mean that the ascorbic acid stimulated matrix formation had but little or no effect on calcification.

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Adsorption of Myxoviruses on Magnetic Iron Oxides. (31020)

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While investigating the phagocytosis of iron oxide particles by cell cultures, it was observed that addition of powdered Fe_2O_3 to allantoic fluids containing each of several myxoviruses caused a decrease in the infective titer of the supernatant and the virus was found to adhere to the rapidly settling metallic oxide. By placing a magnet in the proximity of the bottom or sides of a glass vessel, it was a simple matter to collect the magnetic oxide on the lower surfaces and remove the supernatant fluids. The procedure could be repeated at will to wash the virus. This did not destroy infectivity, and, depending upon the agent used, loss of virus or antigen from the iron oxide was negligible. When it was found that separation of concentrated viral antigen from the complex could be readily accomplished by appropriate treatment, this reaction became one of general interest and is described below.

Materials and methods. Iron oxide. Powdered, acicular or cuboidal Fe_2O_3 (hematite) was obtained from the C. K. Williams Division of Chas. Pfizer & Co., Inc. It was further pulverized by grinding in a ball mill with distilled water for 4 hours to an average particle size of 0.5-1 μ. The final thick slurry was centrifuged to remove excess water and an aliquot dried at 100°C for 20 hours to determine dry weight. Stock suspensions of the wet slurry containing 50 mg/ml were made in distilled water and autoclaved to sterilize. Resuspension of dried Fe_2O_3 powder in water was very difficult and pipetting of aqueous iron suspensions must be performed rapidly in order to secure even distribution of the rapidly settling material.

Viruses. Six representative, egg-adapted strains of influenza virus were grown in the chorioallantoic fluids of 9-day-old embryonated eggs for 48-72 hours and the freshly