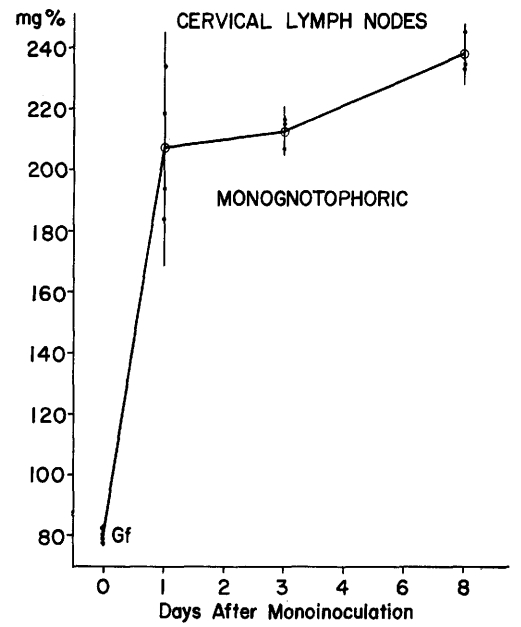

FIG. 4. Weight of combined cervical lymph nodes per 100 g body weight in white mice. The monognotophoric mice were germfree before being inoculated at 0 days on the abcissa. See Fig. 1 for explanation of the symbols.

5-fold increase of the size of ileocecal nodes one week after gross contamination.

Important questions are raised by the observation of the rapidly developing lymphatic tissue. What do the bacteria provide to evoke such dramatic response? How fast do these develop following inoculation? What other microorganisms will do this? What other defense mechanisms are stimulated? The ease with which answers to such questions may be approached illustrates the power of gnotophoric techniques(1) in the study of defense mechanisms.

Summary. Inoculation *of Streptococcus sp.* into germfree rats resulted in dramatic cecal changes within 24 hours. The weight of the cecum and its contents were reduced almost 50% and an apical aggregate of lymphatic tissue appears as a grossly visible entity, lacking only the more densely clumped nature of that found in classic rats. Exploratory work with mice confirmed this observation. Although the size (weight or surface area) of the cecal wall of the germfree rats is twice the size of that in classic rats, no change was noted when germfree rats were monoinoculated with *Streptococcus sp.* The cervical lymph nodes increased 3-fold following inoculation of germfree mice. This exploratory work illustrates the potential of gnotophoric animals in the study of defense mechanisms.

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Factors Controlling Calcification *in vitro*: The Calcium Phosphate Ratio.* (29325)

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In a very early study of calcification processes, Robison, employing epiphyseal cartilage slices from rachitic rats, concluded that the calcium concentration was relatively more

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important than the phosphate concentration in determining whether mineralization would occur *in vitro*(1). Since these early experiments, the importance of the Ca/P ratio has not been systematically studied despite many advances in our understanding of nucleation and the induction of crystal formation in the aqueous calcium phosphate system *in vitro* (2).

Physiologically, the level of serum calcium ion is quite closely regulated while serum phosphate concentrations vary considerably (3). This fact has been utilized in most experiments *in vitro, i.e.* calcium concentration has been kept constant while phosphate concentrations were varied systematically. This automatically introduces the Ca/P ratio as a variable.

In the present studies, the importance of the Ca/P ratio was examined under 3 sets of conditions: a) spontaneous precipitation or "self-seeding" solutions, b) solutions seeded with small amounts of preformed apatite crystals, and c) solutions seeded with bits of extracted tendon, a highly collagenous preparation.

Methods. As in earlier studies(4,5), 3 solutions were prepared: One containing calcium ion, one containing phosphate ion, and one containing KCl and barbital buffer only. Each were made to have an ionic strength of 0.16 and a pH of 7.4 so that they could be mixed in any proportions without changing these important factors in the formation of calcium phosphate salts. After a series of such mixtures were prepared, they were incubated 5 days at 37°, then the crystals were separated from the solution for direct analysis rather than by noting a drop in the solution concentrations as in previous reports.

To study the effect of the calcium phosphate ratio, 3 series of calcium phosphate solutions were prepared, each with increasing calcium phosphate concentration-products but with the ratio of calcium to phosphate constant within any one series. The products of the concentrations were such that the low ones did not form detectable crystals and the high ones did. The maximum product which did *not* form crystals (critical product) was used as the end point since ideally it would give a measure of nucleation ability, independent of the number of nucleating sites or rate of growth of the crystals. A comparison was then made of the critical products obtained in 3 series of solutions prepared with different calcium to phosphate ratios. If the concentrations of calcium and phosphate were equally important in crystal formation then the critical product should not change with varying Ca/P ratios. When changes occur, the relative importance of the 2 ions can be evaluated by solving the following equation for "y": $(Ca^y \times PO_4)_a = (Ca^y \times PO_4)_b$ where the values for Ca and PO_4 are the concentrations at the critical product for the respective series with a Ca/PO_4 ratio "a" and "b." The value of "y" then can be compared with those obtained with different nucleators.

Nucleation was induced by 3 different means: "spontaneous," extracted tendon, and preformed hydroxyapatite crystals. The extracted tendon was largely from the same batch of "collagen" used by Fleisch and Neuman(5). Approximately 5 mg of the tendon in 3 to 5 pieces were used per flask. Analysis of the tendon showed 0.46 micromoles of EDTA titrable cation and 1.46 micromoles of phosphate per 100 mg. These quantities are not thought to be responsible for the formation of crystals directly since: (a) the number of carboxyl groups in the collagen after the extraction to remove the soluble proteins and mucopolysaccharides would greatly outnumber the calcium ions present, making the migration necessary to form crystals difficult; (b) a similar batch of tendon with little if any nucleation ability had the same amount of calcium and phosphate; (c) ashing the tendon destroyed nucleating ability; and (d) this slight contamination did not significantly (ca. 0.2%) affect the Ca $\times$ PO_4 product or its ratio. However, since bovine achilles tendon is the source of the extracted tendon and can be expected to contain 5% elastin(6) which would not be expected to be removed by the extraction procedure, it cannot be assumed that collagen *per se* is necessarily the active nucleating principle present.

The preformed hydroxyapatite crystals were from a large batch that has been charac-

terized by several investigators, summarized by Neuman(7), and more recently, additional information has been given by Rootare *et al* (8). The crystals were suspended at room temperature in the solution used for dilution of the mineralizing solutions. The suspension was allowed to settle for 2-3 hours so that the larger aggregates were removed, then a portion of supernatant was removed for use in the experiments. The solid in the 0.3 ml of suspension (added per 30 ml flask) contained approximately 0.06 micromoles of calcium or 2 to 3 times the amount present in the tendon used. The amount of calcium and phosphate in solution in the added suspension changed the mineralizing solutions to which they were added less than 0.3%, and was ignored.

Direct analysis of the small amounts of solid phase required removal of the contaminating solution. In the case of the spontaneous precipitation and with the preformed crystals the flask contents and one 30 ml rinse were passed through a 450 mμ filter. The extracted tendon was removed from the solution and blotted. The filter papers and the pieces of tendon were ashed and analyzed while the crystals remaining in the flasks were dissolved and analyzed directly. In the case of spontaneous and crystal-induced samples the value from the filter and flask were added but not in the case of the tendon. The only time the flasks seeded by tendon showed evidence of crystals on the walls of the flask itself was when the calcium phosphate product was high enough to cause spontaneous precipitation.

To determine the amount of crystal lost by rinsing, 10 ml aliquots of a suspension of hydroxyapatite were filtered through pairs of filters without rinsing. The amount on the top filter (450 mμ) minus that found on the one underneath was taken as the amount of crystal present in the suspension (0.9 micromoles as phosphate). Ninety-five percent recovery was observed when rinsed with the same quantity of water used in the experiments.

Calcium analysis was performed by direct EDTA titration with calcein indicator(9) of the dissolved ash or crystals. To avoid the sluggish end point caused by the phosphate most of the EDTA was added before the KOH. The amount needed was estimated by prior visual observation of the amount of crystal present. Phosphate was determined on the same solution (after neutralization of the KOH) by adding an equal volume of phosphate reagent, described by Chen *et al* (10), and then diluting to a specific volume after color development.

Since the crystals adhered to the walls of the flasks it was thought the type of container might be an important factor in initiating crystals, hence polyethylene containers were also used, with no apparent difference.

Results. Fig. 1 shows the amount of solid or crystal, expressed as micromoles of phosphate, formed by spontaneous precipitation *vs* the calcium phosphate concentration product in the solution at the beginning of experiment. The individual lines represent a series of solutions at the Ca/PO_4 ratios indicated by the adjacent numbers. The 3 lines do not coincide. Rather, calcium appears more effective than phosphate in inducing crystal formation. Calcium has to be raised to some exponent in order for a constant critical product to be operative. A value of $1.6 \pm .02$ for the exponent, y, fitted the 3 sets of data.

The results with extracted tendon as a nucleator are shown in Fig. 2. Here it is seen that crystallization occurred at lower ion products. Furthermore, the effect of the ratio, Ca/PO_4, was much less marked, $y = 1.1$ to 1.3.

Fig. 3 shows the behavior of the preformed crystal in the 3 series of solutions. It shows that the product needed to cause detectable growth of the crystals is about the same as for the tendon but changes in the calcium phosphate ratio produced more effect. The value of y was 1.6 to 1.9.

The formation of crystals on the extracted tendon involves both nucleation and crystal growth. Assuming, as seems reasonable, that the growth phase is dominant in the portion of the curves where considerable solid is formed it should be possible to determine a second value, y, that corresponds to the value found with preformed crystals. Using the

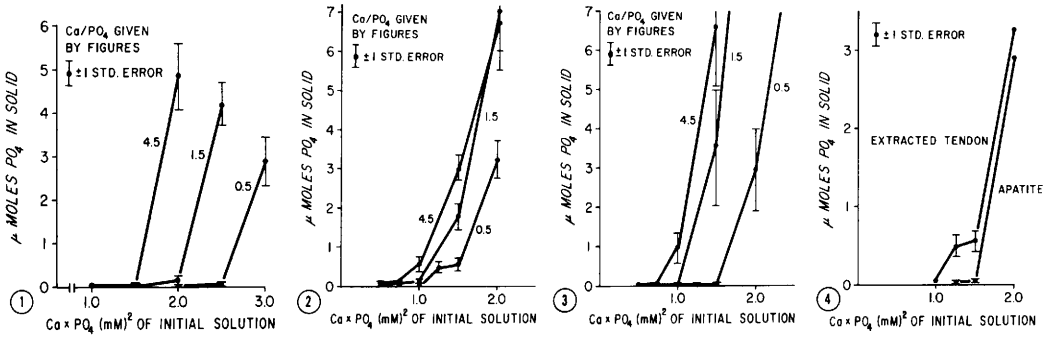

FIG. 1. Effect of changing calcium phosphate ratio in solutions with increasing calcium phosphate products with no added nucleator present. Amount of crystal formed in 5 days at 37°C is given by the ordinate expressed as PO_4. Calcium content was 1.5 to 1.6 times as much.

FIG. 2. Effect of changing calcium phosphate ratio in solutions with increasing calcium phosphate products with extracted tendon added.

FIG. 3. Effect of changing calcium phosphate ratio in solutions with increasing calcium phosphate products with small amounts of preformed hydroxyapatite crystals added.

FIG. 4. Comparison of amount of solid formed when the solutions had a calcium phosphate ratio of 0.5 and seeded with extracted tendon vs preformed hydroxyapatite crystal. Amount of calcium in the crystals was greater than that present in the extracted tendon. See text for behavior when more nucleator is present.

segments of the curves from 0.5 to 3.0 μmoles phosphate and projecting them back to the base line the value for "y" is approximately 1.6.

Fig. 4 is a comparison of the extracted tendon with the preformed crystals when both were incubated in solutions with a ratio Ca/P of 0.5. The extracted tendon is seen to be superior to this quantity of preformed crystal which was twice the amount that could be present as a contaminant on the extracted tendon. Additional studies were performed in which 10 times as much tendon and preformed crystal were used. In such cases the superiority of tendon was lost. The breaking point in the curve using more tendon did not shift whereas with increased amounts of crystal it shifted to lower products (with either high or low Ca/PO_4 ratios). The angle of the lines at the critical point became more acute with more tendon and less acute with more crystal so that comparison of the critical products became increasingly difficult.

The calcium phosphate ratio of the solid phase formed with spontaneous precipitation was 1.5 to 1.6 depending on the ratio of the solution in which it was formed. The ratio formed with the tendon was 1.62 ± 0.06 (S.D.) with no apparent dependence on the solution ratio.

Discussion. The data demonstrate experimentally a clear difference between precipitation and crystal-seeded solutions on the one hand and organic-catalyzed crystal formation on the other. In the absence of extracted tendon, the critical product was very sensitive to the Ca/PO_4 ratio of the solution. Unseeded solutions gave a value of 1.6 for the exponent, y, in $(Ca)^y \cdot (PO_4) = K$, crystal-seeded solutions a value of 1.6 to 1.9. In contrast, tendon-seeded solutions were relatively insensitive to variations in the Ca/PO_4 ratio, the value of the exponent, y, being close to unity, 1.1-1.3.

Robison many years ago studied the effect of varying Ca/P ratio on the mineralization of epiphyseal slices from rachitic rats(1). An analysis of his data gave, as a best fit, a value of the exponent, y, of 1.2.

The proper exponent for expressing the serum calcium times phosphate in evaluating the status of rickets was debated many years ago, but was never resolved. Shear and Kramer insisted that clinically Ca times P was the best expression(11) even though Holt had claimed that $(Ca)^3$ times $(PO_4)^2$ was better in judging the same data as well as being theoretically more sound(12). The data presented here suggest that perhaps both were correct to some extent. The amount of inorganic salts deposited in an organic matrix

presumably would be determined by both nucleation and crystal growth and since the values of the exponent obtained for the extracted tendon were approximately 1.2 and 1.6 for the 2 respective processes both points of view can claim some support.

If initiation of crystals in matrices *in vivo* does indeed follow the same pattern then evolution has selected an advantageous homeostatic regulation. Calcium concentrations throughout life are essentially constant, while phosphate concentrations are relatively high during the period of rapid skeletal development in early life. Such a situation would promote mineralization of an organic matrix without a corresponding increase in the risk of spontaneous or aberrent, uncontrolled precipitation.

The findings for the preformed crystals were particularly unexpected and provocative, suggesting that there is a sharp break in rate of growth well above the solubility equilibrium value and that a second phase becomes important with the higher products. However, this breaking point shifts with the amount of crystal charge and while additional work has ruled out problems of recovery or traces of inhibitors as explanations(13) further work is necessary before conclusions may be drawn. These data are included here because the findings are not consistent with possible contaminating crystals on the extracted tendon as an explanation for the relative insensitiveness of tendon nucleation to changes in the calcium phosphate ratio.

A possible reason for the difference between the extracted tendon and the other nucleators may lie in calcium binding. If the density of bound calcium is important, and there are a limited number of sites, then variations in calcium concentration in the solution become comparatively less important. Raising the calcium concentration becomes less effective in increasing the density of calcium on the nucleator as the binding sites are saturated.

Summary. 1. Altering the calcium phosphate ratio of calcifying solutions has little effect on the initiation of crystals by an organic matrix in contrast to "spontaneous" precipitation, and the growth of crystals. 2. This observation sheds light on the unresolved argument concerning the old clinical impression that the simple calcium phosphorus product was the best estimate of healing rickets in contrast to that expected theoretically from the calcium phosphate ratio of the salt formed. 3. A difference of this kind would be biologically advantageous since elevation of the serum phosphate would increase the mineralization of seedable matrices more than the tendency toward uncontrolled precipitation.

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