Effect of Beryllium on *In Vitro* Calcification of Cartilage. (18239)

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"Beryllium rickets"(1-6) has generally been ascribed to the marked hypophosphatemia resulting from diminished absorption of phosphate from the gastro-intestinal tract due to precipitation of beryllium phosphate in the gut. There are indications, however, that beryllium also adversely affects the so-called local factor in calcifying cartilage(4). Thus administration of vitamin D will restore the serum inorganic phosphate to normal levels but nevertheless appears to be incapable, even in high dosage, of wholly preventing or curing the abnormality in endochondral calcification(4,5). Moreover, Sobel(4,7) found that the cartilage of rats fed a beryllium-containing diet would calcify only very imperfectly *in vitro* in solutions containing adequate concentrations of calcium and inorganic phosphate. This latter finding is of interest in connection with recent evidence(8-12) that beryllium in very low concentration markedly inhibits alkaline phosphatases, being more selective in this respect than cyanide(13-15), formaldehyde(16) or any other chemical or physical agent yet employed. The aim of the present study was to exploit this property of beryllium for further investigation of the role of enzymes, in particular alkaline phosphatase, in endochondral calcification.

Methods. Twenty-day-old rats were placed on the Schneider-Steenbock rachitogenic diet R-14 for 7-10 days to produce moderately advanced rickets and then sacrificed. For our preliminary studies of Be inhibition of cartilage alkaline phosphatase activity, the epiphyseal cartilage of the tibiae and femora of 2 rats was dissected out for each experiment, ground with sand in 10 ml 0.04 M veronal buffer, pH 9.8, and the homogenate then centrifuged. The phosphatase activity of the samples was then standardized by diluting the slightly turbid supernatant with veronal buffer (usually 3-5 ml supernatant diluted to 10 ml) to give enzyme activity sufficient to split off 10-20 γ P as inorganic phosphate by a 0.2 ml sample after one-half hour incubation with 1% sodium β-glycerophosphate. In experiments to determine percent inhibition by various molarities of beryllium, 0.8 ml beryllium sulfate in concentrations prepared to give the indicated final molarities was added to tubes containing 0.2 ml standardized cartilage extract and 1.0 ml β-glycerophosphate-veronal buffer-substrate solution. The mixture was incubated for 30 minutes at 37°C. (Control tubes were similarly prepared but without added beryllium). After cooling in an ice-bath, 2 ml 10% trichloroacetic acid was added and inorganic P determined by the Fiske and Subbarow method adapted to photoelectric colorimetry. Time-activity experiments were carried out in the same way except for varying the time

1. Guyatt, B. L., Kay, H. D., and Branion, H. D., *J. Nutrition,* 1933, v6, 313.

2. Jacobson, S. A., *Arch. Path.,* 1933, v15, 18.

3. Kay, H. D. and Skill, D. I., *Biochem. J.,* 1934, v28, 1222.

4. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.,* 1935, v108, 395.

5. Jones, J. H., *Am. J. Physiol.,* 1938, v124, 230.

6. Maynard, E. A., Downs, W. L. and Scott, J. K., *Fed. Proc.,* 1950, v9, 338.

7. Sobel, A. E., *Trans. Second Macy Foundation Conference on Metabolic Interrelations,* 1950, in press.

8. Cloetens, R., *Biochem. Z.,* 1941, v310, 42.

9. Klemperer, F. W., Miller, J. M. and Hill, C. J., *J. Biol. Chem.,* 1949, v180, 281.

10. Grier, R. S., Hood, M. B., and Hoagland, M. B., *J. Biol. Chem.,* 1949, v180, 289.

11. DuBois, K. P., Cochran, K. W., and Mazur, M., *Science,* 1949, v110, 420.

12. Cochran, K. W., Mazur, M., and DuBois, K. P., *Fed. Proc.,* 1950, v9, 264.

13. Cloetens, R., *Enzymologia,* 1939, v6, 46.

14. Bodansky, O., *J. Biol. Chem.,* 1949, v179, 81.

15. Gutman, A. B., and Jones, B., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 572.

16. Gould, B. S., *J. Biol. Chem.,* 1944, v156, 365.

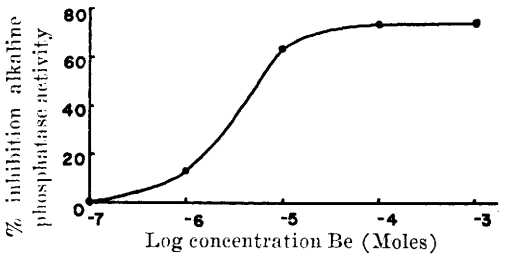

FIG. 1.

Percent inhibition of cartilage alkaline phosphatase activity by Be in various concentrations. (Buffer - substrate: β-glycerophosphate - veronal; final pH 9.6; time ½ hr; temp. 37°C).

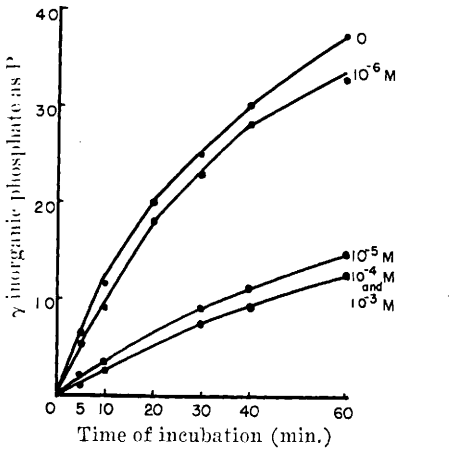

FIG. 2.

Time-activity curves for inhibition of cartilage alkaline phosphatase activity by Be in various concentrations. (Buffer-substrate: β-glycerophosphate-veronal, final pH 9.6; temp. 37°C).

of incubation from 5-60 minutes. The data incorporated in Fig. 1 and 2 represent the means of 8 and 3 sets of runs respectively. For the *in vitro* calcification experiments, contiguous slices of the proximal ends of moderately rachitic rat tibiae were used in controlled sets of experiments conducted as previously described(17). The basal salt solution employed contained potassium chloride 50 meq., sodium chloride 700 meq., sodium bicarbonate 220 meq. per liter, no added magnesium. The calcifying solutions contained in addition Ca (10 mg % as calcium chloride) and P as inorganic phosphate (6 mg %) or as phosphoric ester of the type and in the amount specified in the text. Beryllium sulfate was added to the appropriate calcifying tubes in amounts sufficient to give the indicated final molarities. The pH of the incubating solutions was adjusted to 7.4, checking with a Cambridge glass electrode pH meter. The slices were incubated 18-24 hours at 37°C, then stained with 3% silver nitrate. The results reproduced in Fig. 3 are representative of 5 uniform experiments of each type illustrated.

Results. Inhibition of cartilage alkaline phosphatase activity by beryllium. As indicated in Fig. 1, beryllium in concentrations of 1.0 x 10^{-5}M or greater markedly inhibits cartilage alkaline phosphatase activity, approximately 70% inhibition being noted at the end of the half-hour incubation period. In concentrations of 1.0 x 10^{-6}M beryllium, inhibition is sharply decreased, to a mean of approximately 12% under the conditions of the experiment, and at lower Be concentrations inhibition is negligible. These results with rat cartilage alkaline phosphatase are in good agreement with those reported by Klemperer *et al.*(9) for the range of beryllium concentrations effective in inhibiting hog kidney alkaline phosphatase. The data are also in general agreement with those reported by Grier *et al.*(10) for the effects of various molarities of Be on rat bone, sarcoma and intestinal alkaline phosphatases, although their figures suggest a more uniform slope in Be concentration - phosphatase inhibition curves than indicated by the data of Klemperer *et al.*(9) and of the present authors.

Fig. 2 is a time-activity curve which again emphasizes the critical difference in inhibition of cartilage alkaline phosphatase activity between the ranges 1.0 x 10^{-6}M and 1.0 x 10^{-5}M Be. It is apparent from these curves that enzyme inactivation occurs within the first 5 minutes of incubation, probably because of replacement of Mg by Be, according to evidence suggesting competition between these 2 ions(9,10).

Effect of beryllium on in vitro *calcification with phosphoric esters. In vitro* calcification experiments were carried out with β-glycerophosphate as the sole source of phosphorus,

17. Gutman, A. B., and Yü, T. F., *Trans. First Macy Foundation Conference on Metabolic Interrelations*, 1949, v1, 11.

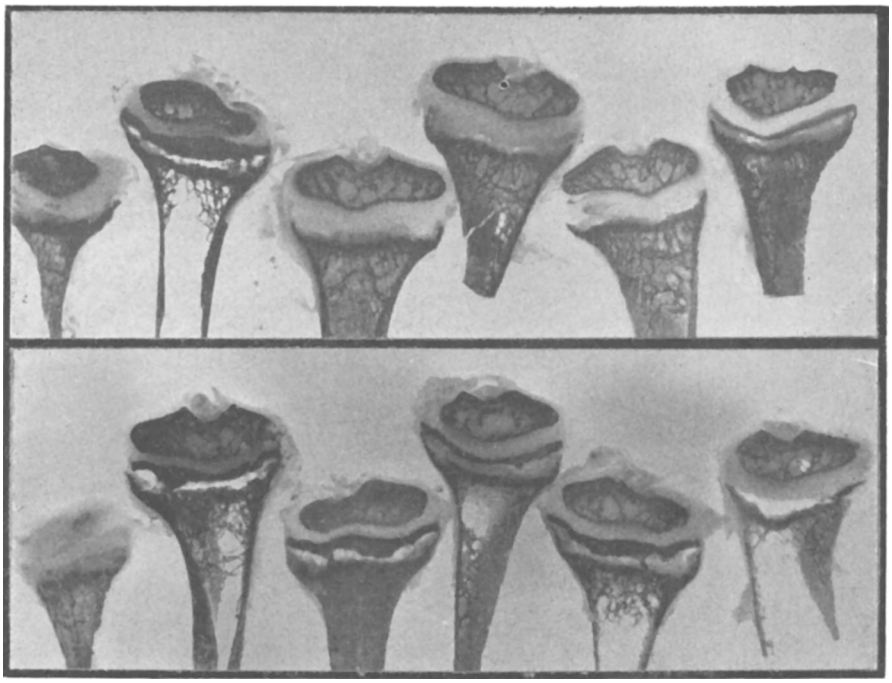

FIG. 3.

Top row, from left to right: 1. Control slice incubated in basal salt solution (B.S.S.) without Ca or P; note wide zone of uncalcified cartilage. 2. Control slice incubated in B.S.S. + Ca + β-glycerophosphate (10 mg % P); note good calcification. 3,4,5,6. Slices incubated in B.S.S. + Ca + β-glycerophosphate (10 mg % P) + Be 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, respectively. Note complete inhibition of calcification in slices 3, 4, and 5, but definite calcification with only partial inhibition in slice 6.

Bottom row, from left to right: 1. Control slice incubated in B.S.S. without Ca or P; note wide zone of uncalcified cartilage. 2. Control slice incubated in B.S.S. + Ca + inorganic P (6 mg %); note good calcification. 3,4,5,6. Slices incubated in B.S.S. + Ca + inorganic P (6 mg %) + Be 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, respectively. Note no inhibition of calcification in slices 3, 4, and 5, but complete inhibition in slice 6.

in concentrations equivalent to 10 mg % P, in the absence of beryllium and in the presence of 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M Be. Good calcification obtained with β-glycerophosphate was completely inhibited in all dilutions of added beryllium up to 10^{-6}M (Fig. 3). In the presence of 1.0 x 10^{-6}M Be, definite calcification was noted although some inhibition was present (Fig. 3). Similar results were obtained when sodium phenylphosphate, in concentrations equivalent to 10 mg % P, and creatine phosphate, in concentrations equivalent to 20 mg % P, were present as the sole source of phosphorus. Calcification was inhibited completely, or virtually so, in all dilutions of beryllium up to 10^{-6}M, in which concentration inhibition was slight and definite calcification was noted. *In vitro* calcification experiments with β-glycerophosphate in higher concentration, equivalent to 20 mg % P, revealed that increasing the substrate tended to decrease the inhibitory effect of beryllium; there was no indication of inhibition by 10^{-6} or 10^{-5}M Be but there was definite inhibition by 10^{-4} and complete blocking by 10^{-3}M Be. That increasing concentrations of phosphoric ester substrate decrease beryllium inhibition of dephosphorylation by alkaline phosphatase was noted by Klemperer(9) using various concentrations of phenylphosphate.

Effect of beryllium on glycolysis of cartilage. Manometric studies of glycolysis by rat cartilage homogenates in Ringer-bicarbonate solution indicated no inhibition by beryllium in

concentrations of 1.0 x 10^{-3}M. This finding is consistent with the report by Klemperer (2) that glycolysis by acetone liver or kidney preparations is not inhibited by beryllium in concentrations up to 10^{-3}.

Discussion. There is a striking correspondence between the concentrations of beryllium which markedly inhibit cartilage alkaline phosphatase and those required to block *in vitro* calcification of cartilage when phosphorus is supplied as β-glycerophosphate, phenylphosphate or creatine phosphate. This finding is consistent with Robison's theory of the role of alkaline phosphatase in making the phosphorus of phosphoric esters available for calcification of cartilage(18). On the other hand, it is difficult to reconcile with the Robison theory the failure of beryllium (except in concentrations of 10^{-3}M) to inhibit *in vitro* calcification of cartilage when phosphorus is supplied as inorganic phosphate. There are other indications that the enzyme mechanisms involved in *in vitro* calcification of cartilage are not the same when the source of phosphorus is β-glycerophosphate, phenylphosphate or creatine phosphate as when phosphorus is supplied as inorganic phosphate. Thus, 10^{-3}M iodoacetate, 10^{-4}M fluoride and 10^{-2} phlorizin, which do not inactivate alkaline phosphatase, do not inhibit *in vitro* calcification of cartilage with β-glycerophosphate, phenylphosphate or creatine phosphate(17, 19,20) but markedly inhibit calcification with inorganic phosphate(17-19). These effects of phlorizin, iodoacetate and fluoride, well known inhibitors of glycogenolysis, form the basis for the view(17,19.20) that phosphorylative glycogenolysis is an integral part of the complex processes of calcification of cartilage in a medium containing phosphorus as inorganic phosphate in physiological concentrations. According to this concept the role of alkaline phosphatase in the hypertrophic cartilage cell does not at present appear to be essential for calcification, at least as a dephosphorylating enzyme, and remains obscure(20).

It will be noted that *in vitro* calcification of cartilage with inorganic phosphate was completely inhibited by beryllium in concentrations of 10^{-3} (Fig. 3). In this concentration beryllium can no longer be regarded as a highly selective inhibitor of alkaline phosphatase. We cannot, however, ascribe the effect on calcification with inorganic phosphate to inhibition of the glycolytic system of enzymes in cartilage and the enzyme system blocked by 10^{-3}M Be remains to be identified.

In regard to the nature of "beryllium rickets," our *in vitro* calcification experiments bear out the contention of Sobel, Goldfarb and Kramer(4) that beryllium interferes with the function of the local factor. The nature of this interference remains obscure but presumably some enzyme essential for calcification is inhibited. Our studies suggest that inhibition of cartilage alkaline phosphatase will not satisfactorily account for the failure of calcification under these circumstances.

Summary. 1. Beryllium in concentrations of 1.0 x 10^{-5}M or greater is a potent inhibitor of the dephosphorylating activity of cartilage alkaline phosphatase. 2. In concentrations of 10^{-5}M or greater, beryllium completely blocked *in vitro* calcification of cartilage when phosphorus was supplied as β-glycerophosphate, phenylphosphate or creatine phosphate. 3. When phosphorus was supplied as inorganic phosphate (the condition apparently obtaining *in vivo*), concentrations of beryllium sufficient to produce marked inhibition of alkaline phosphatase failed to interfere with *in vitro* calcification of cartilage. 4. The significance of these findings in relation to current theories of endochondral calcification and "beryllium rickets" is discussed.

18. Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, v28, 694.

19. Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, v95, 461.

20. Gutman, A. B. and Yü, T. F., *Trans. Second Macy Foundation Conference on Metabolic Interrelations*, 1950, in press.

21. Klemperer, F. W., *Fed. Proc.*, 1950, v9, 190.

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