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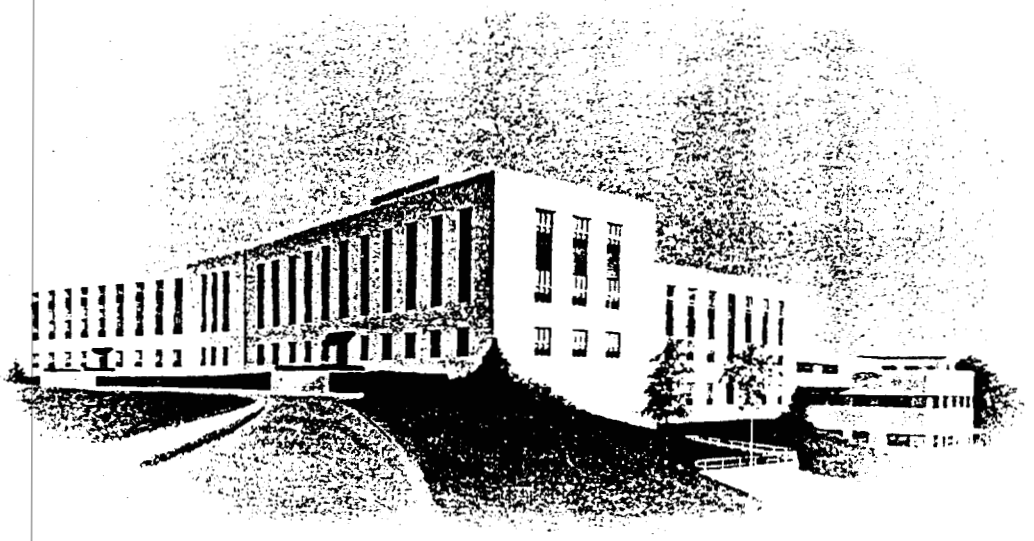

STUDIES OF THE METABOLISM OF GALLIUM, IV.
EFFECT OF GALLIUM ON ALKALINE PHOSPHATASE AND CALCIFICATION *IN VITRO*

RESEARCH REPORT
Project NM 007 081.06.13

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STUDIES OF THE METABOLISM OF GALLIUM, IV.
EFFECT OF GALLIUM ON ALKALINE PHOSPHATASE AND CALCIFICATION *IN VITRO*

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ABSTRACT

Studies of the influence of gallium on cartilage alkaline phosphatase *in vitro* show that there is a gallium concentration range (2.50×10^{-6} to 1.0×10^{-5}M) which markedly increases the activity of this enzyme. Higher gallium citrate and equimolar sodium citrate concentrations prevent the reduction of phosphomolybdic acid in the Fiske-Subbarow method.

By Von Kossa's silver staining technique, *in vitro* calcification of rachitic rat tibias and femurs have been studied. With 5.0×10^{-6}M gallium there appears to be a slight increase in calcium deposition over the equimolar sodium citrate control.

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INTRODUCTION

Earlier studies of the chemical nature of the local factors which effect selective deposition of gallium in the bone (1, 2) led to the belief that gallium may influence the mechanism of alkaline phosphatase activity in the calcification process in bone. This report presents the results of a further investigation of the biochemical action of gallium as related to the calcification process in the bone.

Robinson (3, 4) has suggested that the calcifying mechanisms were partly enzymatic; specifically that the alkaline phosphatase in hypertrophic cartilage acted upon some phosphoric ester substrate present to produce inorganic phosphate which resulted in a condition of local supersaturation and precipitation of the bone salt in the presence of Ca^{++}. Yii and Gutman (5, 6) further showed that beryllium inhabited alkaline phosphatase activity and *in vitro* calcification. In view of the preceding evidence of beryllium inhibition of alkaline phosphatase in very low concentrations, the role of gallium was studied.

PROCEDURES

A. Enzyme Activity Determination

Sprague-Dawley rats of both sexes, 40 to 50 days old were used. The tibial and femoral bones of these rats were removed and stripped free of muscle tissue. The section between and including the epiphyseal cartilage of the upper extremity of the tibia and lower extremity of the femur were dissected free and used for enzyme source. Tibial and femoral bone sections from rats, three to a group, were homogenized by mortar and pestle with chemically pure ground sea sand in 10 ml. of 0.04 M veronal buffer, pH 9.70. The homogenate was then centrifuged, the supernatant (usually 3 to 5 ml.) decanted and diluted to 10 ml. with veronal buffer.

Two-tenths (0.20) ml. aliquots of the enzyme mixture were added to 1 ml. of 1 percent sodium B-glycerophosphate at pH 8.90 and the volume made up to 2 ml. with 0.80 ml. of double distilled water or test solutions. These mixtures were incubated for 30 minutes at 37° C., cooled in an ice bath, and 2 ml. of 10 percent trichloracetic acid was added. The enzyme precipitate was then removed by paper filtration, and the inorganic phosphorus in the filtrate measured colorimetrically by the Fiske-Subbarow method. (7)

Only those enzyme preparations were used which contained sufficient activity in an 0.20 ml. aliquot to liberate 10 to 20 micrograms of phosphorus.

Preliminary experiments were carried out to test the effect of the citrate and gallium ions on the color formation by the reduction of phosphomolybolic acid in the Fiske-Subbarow method. It was found that the citrate ion in concentrations used with 1.0×10^{-4} M of gallium and greater amounts of 5.0×10^{-4} M, inhibited color formation. The citrate ion used with gallium concentrations of 5.0×10^{-5}, 5.0×10^{-6}, and 5.0×10^{-7} M has no effect on the color production, nor does the gallium ion at concentrations of 5.0×10^{-5}, 5.0×10^{-6}, and 5.0×10^{-7} M.

A citrate control was used in each experiment to determine the effect of the citrate ion on the reaction. Sodium citrate pH 9.00 was used in the same molar concentrations as was used in the gallium citrate complex. Sodium in these experiments was considered to be inert. The effect of gallium [99.98% (8)] on enzyme activity was tested using the same procedure, but substituting 0.80 ml. of a gallium citrate solution (9) adjusted to a pH 9.00 in varying molar concentrations in place of the double distilled water. Percent; acceleration vs time curves

(ordinate: $\frac{\mu g\,[\text{exptl.}] - \mu g\,[\text{aqueous}]}{\mu g\ \text{aqueous}} \cdot 100$ percent; abscissa: incubation time in minutes)

were obtained by assaying 0.20 ml. aliquots for alkaline phosphatase activity after 10, 20, 30, 40 and 60 minutes' incubation at 37°C. (Fig. 1).

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B. *In vitro* calcification

The influence of gallium on calcification in the tibias and femurs of rachitic rats was studies by using Von Kossa's (10) *in vitro* silver staining technique. Rachitic rats were chosen for this study because of the increased epiphyseal cartilage zone and the greater activity of the alkaline phosphatase in this area.

Twenty-day-old Sprague-Dawley rats (male and female) were started on a rachitogenic diet (No. 2, U.S.P.) and maintained for 20 to 25 days to produce severe rickets. In each experiment the proximal ends of the tibias and femurs from one animal were stripped clean of muscle and adjacent tissue. The four bones were cut longitudinally by free-hand slicing through the center, giving four pairs (2 tibial, 2 femoral) of identical surfaces for comparison. The thin longitudinal sections were washed in physiologic saline solution. Two slices (one each from the tibia and femur) were used for the effect of gallium on calcification and the other six slices (2 without Ca/P, 2 with Ca/P, 2 with Ca/P+Citrate) were used as controls. Six to eight paired experiments were run with each concentration of gallium.

The incubating solutions used for controls and for testing the influence of gallium on calcification consisted of: (a) a basal salt solution (11) (KCl 5 meq., NaCl 70 meq. and $NaHCO_3$ 20 meq.); (b) calcium (9.3 mg. percent Ca as $CaCl_2$); (c) organic phosphate (10 mg. percent P as sodium B-glycerophosphate); (d) gallium citrate (pH 7.35 to 7.40) in varying molar concentrations and (e) sodium citrate (pH 7.35 to 7.40) in equimolar citrate concentrations. One control was run with only the basal salt solution (a) to show the lack of calcification. A second control was run to show the normal amount of *in vitro* calcification occurring with only Ca (b) and P (c) plus the basal salt solution (a). A third control (a,b,c, and e) was used to determine the influence of the citrate ion on calcification. The experimental gallium solution (a,b,c, and d) was used to determine the influence of the gallium ion on calcification.

The pH of these incubating solutions was adjusted to 7.35 to 7.40 (glass electrode) by the cautious admission of oxygen or carbon dioxide gases. Forty ml. of one of the appropriate incubating solutions was used for each slice in a conical flask. The pH was further adjusted to 7.35 to 7.40 by adding 0.10 N HCl or 0.10 N NaOH. The whole solution was saturated with nitrogen (99.5% N and 0.5% CO_2) to establish anaerobic conditions and the flasks stoppered and sealed with adhesive tape. Phenol red was added to detect gross pH changes during incubation.

The slices were incubated at 37° C. for 18 to 24 hours, and were continually rocked (mechanically) within a 90° angle (at a frequency of approximately 10 per min.) to insure uniform surface exposure. At the expiration of the time after incubation, the slices were washed thorcughly in double distilled water and stained by adding 3 percent silver nitrate to a light brown color by exposing to bright diffuse daylight. They were then washed in the dark several times with double distilled water and photographed. The amount of calcification determined by the degree of silver staining graded 0 to +++++ by the method of Marks and Shorr (12).

RESULTS AND CONCLUSIONS

The influence of gallium on cartilage alkaline phosphatase activity of the rat tibial and femoral bone sections showed, with amounts less than 2.5×10^{-6} M, gallium did not result in any increased enzyme activity. The gallium ion increased the phosphatase activity at molar concentrations of 2.5×10^{-6}, 5.0×10^{-6} and 1.0×10^{-5}. With 5.0×10^{-5} M gallium there was a negligible effect on the phosphatase activity. The citrate ion at equimolar concentrations to the gallium ion resulted in a negligible effect on the activity of the enzyme.

The influence of gallium on the degree of *in vitro* calcification in the epiphyseal cartilage was compared with the citrate control. It was found that gallium in concentrations lower than 5×10^{-6} M ranged from no effect with 5×10^{-8} M to slight inhibition with 5×10^{-7} M. Gallium concentrations of 5×10^{-6} M caused a slight increase in calcification. Increased gallium concentrations (5×10^{-5} M) showed inhibition of calcification. These results are shown in Table 1 and Figure 2.

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Though the influence of gallium concentration on *in vitro* calcification was not as marked as was observed in the effect of gallium on phosphatase activity, there was, in general, agreement between the two experiments. Gallium concentrations of $5x10^{-6}$ M produced maximal increase in alkaline phosphatase activity and also the greatest increase in *in vitro* calcification.

Conversely, inhibition in enzyme activity produced by higher concentrations ($5x10^{-5}$ M gallium) was also observed in the calcification experiment. This inhibition may be accounted for on the basis of increased concentrations of the citrate ion (13).

SUMMARY

A study has been made of the influence of gallium on the cartilage alkaline phosphatase *in vitro*. These results indicate that there is a gallium concentration range (2.5×10^{-6} to 1.0×10^{-5} M) which markedly increases the activity of this enzyme.

By a silver staining technique, *in vitro* calcification of rachitic rat tibias and femurs has been studies. With 5×10^{-6} M gallium there appears to be a slight increase in calcium deposition over the equimolar citrate control, whereas with 5×10^{-7} M gallium there is lowered calcium deposition.

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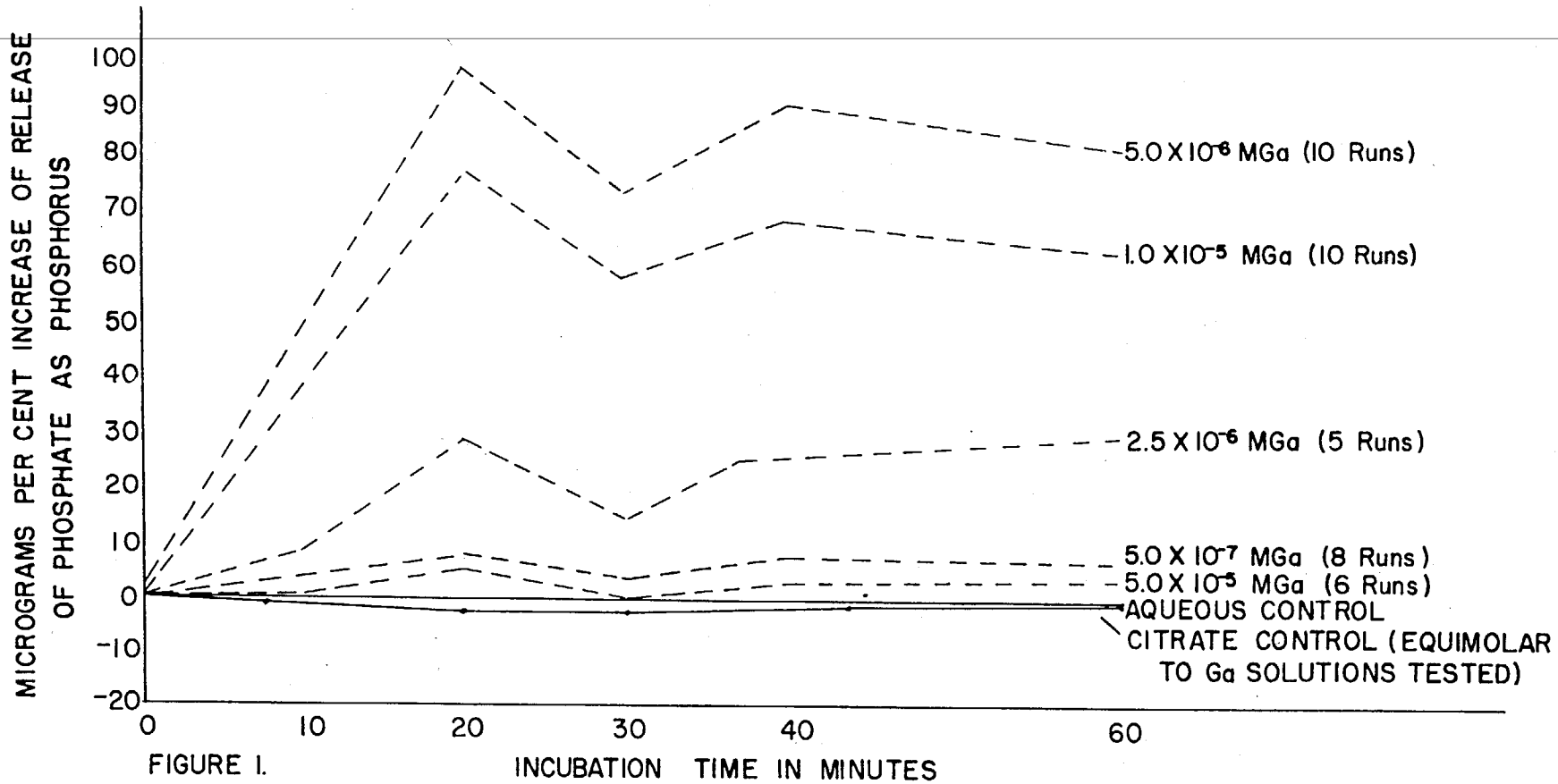

FIGURE I.

TIME—PER CENT ACCELERATION CURVES OF CARTILAGE ALKALINE PHOSPHATE ACTIVITY BY GALLIUM IN VARIOUS MOLARITIES.
(B-GLYCEROPHOSPHATE—VERONAL BUFFER; PH 9.7 TEMP. 37°C.)

Gallium Concentration	Citrate Control	Gallium Citrate	Remarks
5×10^{-5}M			No calcification in epiphyseal cartilage in either slice.
5×10^{-6}M			Increased calcification in epiphyseal cartilage in gallium treated slice.
5×10^{-7}M			Decreased calcification in epiphyseal cartilage in gallium treated slice.
5×10^{-8}M			Calcification in both slices are of the same order

Figure 2: Photographs of Head of Rachitic Rat Tibia Showing Influence of Gallium and Citrate on *in vitro* Calcification.
(Silver nitrate stain; citrate control has equimolar citrate)

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TABLE 1

Effect of Gallium on *in vitro* Calcification* in Rat Epiphyseal Cartilage.

Gallium Concentration	Source of Phosphorus	TIBIA Citrate Control	TIBIA Gallium Citrate	FEMUR Citrate Control	FEMUR Gallium Citrate
$5x10^{-8}M$	Organic Phosphorus	+++ ++++ ++++	+ ++++ ++	+++ +++ +++	lost ++ ++
	Inorganic Phosphorus	+++ ++++ ++++	+++ ++ ++	++ ++++ +++	0 ++++ +++
$5x10^{-7}M$	Organic Phosphorus	+++ +++ +++	0 0 0	++++ +++ ++++	+ + ++
	Inorganic Phosphorus	++ +++ +++	+ 0 ++	++ +++ +	0 + 0
$5x10^{-6}M$	Organic Phosphorus	++ + 0 0	+++ + + ++	+ ++ ++ +	++ +++ +++ ++
	Inorganic Phosphorus	++ +++ ++ 0	++ +++ +++ ++	++ ++ 0 0	+ ++ + 0
$5x10^{-5}M$	Organic Phosphorus	0 + 0 0	++ 0 0 0	0 0 0 0	0 0 0 0
	Inorganic Phosphorus	0 + 0 0	0 0 0 0	0 + 0 0	++ 0 + 0

*Degree of deposition graded from 0 to ++++ after Marks and Shorr (11).

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