CCCXIII. PHOSPHATASE ACTIVITY AND CALCIFICATION IN STRONTIUM RICKETS.

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(Received September 6th, 1935.)

IN view of the great importance attached to the rôle of bone phosphatase in the intimate mechanism of bone formation [Robison, 1932; Kay, 1932; Robison and Rosenheim, 1934; Niven and Robison, 1934; Fell and Robison, 1934] investigations were undertaken to determine whether the remarkably reduced calcifying power of the endochondral cartilage in strontium rickets [Sobel *et al.*, 1934] is associated with a decreased activity of this enzyme. Either a retarding effect of Sr^{++} on the activity of bone phosphatase, or the interference of strontium with the formation or retention of this enzyme in the bone or cartilage cell or both may cause such a change.

With these considerations in mind the effect of various concentrations of Sr^{++} on the activity of bone phosphatase was determined (as measured by the amount of sodium glycerophosphate hydrolysed per hour); no significant change in the activity of the enzyme was observed.

This negative result however did not rule out the possibility of a decreased phosphatase content in the bone cells of animals suffering from strontium rickets when compared with the phosphatase content of the bone cells in calcium rickets. Consequently, the phosphatase content in similar anatomical portions of the tibiae of animals suffering from strontium rickets was compared with that of corresponding portions removed from animals with calcium rickets. In addition, these enzyme values were compared with those of bones removed from animals which had previously developed strontium rickets and which were then changed to the calcium rachitic diet and maintained on this diet until the *in vitro* calcifying property of the bone cartilage was restored.

The results of these investigations have shown no significant differences in the phosphatase contents of the tibiae of the various groups of animals, although there was marked difference between the *in vitro* calcifying powers of the tibiae in the same groups. Incubating the tibiae of the animals suffering from rickets due to strontium in artificial serum solutions with a $\text{Ca} \times \text{P}$ product of 60 for 22 hours failed to show new calcification. In contrast, the control rachitic group showed ample new calcification under identical conditions. The strontium group which was transferred to the calcium rachitic diet showed deposition similar to that observed in the control group.

It is obvious from our results that some factor other than phosphatase activity is altered in the bone cells of animals suffering from strontium rickets. Before the nature of this injury can be understood it will be necessary to understand the process of calcification in the matrix of the bone cartilage cell. It is hoped that further investigations, which are in progress, will throw additional light upon the subject.

Determination of phosphatase activity.

The tibiae were carefully excised. The epiphyseal portion of the bone just above the junction of the fibula was sliced into very small thin sections which were placed in small bottles containing 10 ml. of water saturated with chloroform. After 12 days the bottles were vigorously shaken in a machine for 8–12 hours. The extract was then filtered through a No. 42 Whatman filter-paper. At this stage, following the technique of Bodansky [1933], 10 ml. of substrate were measured into a test-tube 18×200 mm., warmed to 37° and then treated with 1 ml. of the filtered bone extract. The contents were mixed by a single inversion and the tube placed in a water-bath at 37° . After exactly 1 hour the test-tubes were transferred to an ice-bath and 9 ml. of 18% trichloroacetic acid added. The contents were then mixed and filtered through two Whatman No. 42 filter papers. 2.5 ml. of this filtrate were used for the determination of inorganic phosphate following the technique of Fiske and Subbarow [1925] except that the amounts of reagents used and the final volume were halved. The inorganic phosphate content of the bone extract was determined by adding 2 ml. of 20% trichloroacetic acid to 3 ml. of the extract and using 2.5 ml. of the filtrate for the colorimetric determination as outlined above.

The phosphatase activity was expressed as mg. P liberated as inorganic phosphate by each bone in 1 hour.

The technique used for the determination of the phosphatase activity of these bones was acquired after considerable preliminary investigations to determine the best conditions for the extraction of the enzyme. The method finally adopted is such that in our hands there was no measurable enzymic activity left in the residual bone. The greatest source of error lies in excision and subsequent slicing of the tibia; therefore special precautions were taken in both cases. The tibia was excised in such a manner as to exclude any part of the fibular epiphysis, yet completely to include the tibial epiphysis. The slicing was carried out with sharp Bard-Parker blades, after which the blades were washed by dipping them into the chloroformed water used for the extraction of the particular bone.

The p_H of the substrate was 9.5 with our reagents when prepared according to Bodansky [1933]. When p_H 8.8 was desired the adjustment was made by adding hydrochloric acid. No change of p_H was observed after the hydrolysis; this is to be expected because only a small fraction of the substrate is hydrolysed.

Measurements of phosphatase activity were made at p_H 9.5 and 8.8. Parallel determinations at both reactions were carried out in some cases on aliquot samples of the same bone extract. The mean ratios¹ of 14 parallel determinations are presented in Table I.

Table I. *Comparative phosphatase activities at p_H 8.8 and 9.5.*

No. of bones 14.			
Relative bone phosphatase activity	$\frac{p_H \text{ 8.8}}{p_H \text{ 9.5}}$	$= 0.640 \pm 0.0048$	
„	„	$\frac{p_H \text{ 9.5}}{p_H \text{ 8.8}}$	$= 1.56 \pm 0.013$

The results of a series of observations all through this paper are expressed as the mean value $\pm$ probable error of the mean.

The effect of strontium ions on the activity of bone phosphatase.

To 10 ml. of substrate (p_H 8.8) various amounts of Sr^{++} in the form of $SrCl_2$ were added. The p_H was adjusted to 8.8 by suitable addition of alkali. The final volume was made up to 12 ml., to which 1 ml. of bone extract was added. The relative phosphatase activities are shown in Table II.

Table II. *Relative phosphatase activities in the presence of various amounts of Sr^{++} .*

mg. of Sr^{++} in 100 ml. of hydrolysis mixture	0.0	0.2	1.0	4.0	20.0	80.0
Relative phosphatase activity	1.00	0.99	0.97	0.98	1.01	1.11

It can be readily seen from the table that much larger amounts of strontium than are likely to occur physiologically do not interfere with the activity of the enzyme.

Comparative bone phosphatase studies.

Albino rats raised in our laboratory from an original Wistar strain were used. The mothers were kept on the stock diet of Bills *et al.* [1931]. The young were ordinarily weaned at 21 days at which time they were placed on the stock diet. At the age of 23–24 days, this was replaced by the experimental diet which was a modified Steenbock-Black [1925] rickets-producing diet consisting of a basal regimen with addition of Ca or Sr as follows:

Basal diet: Corn meal 71 parts

Wheat gluten 20 parts

Brewer's yeast (Mead's) 5 parts

NaCl 1 part

Ca rachitic diet: Basal diet 97 parts + 3 parts of $CaCO_3$

Ca = 1.2 % = 30.0 mg. mol./100 g. P = 0.14 % = 45 mg. mol./100 g.

Sr rachitic diet: Basal diet, 97 parts + 3 parts of $SrCO_3$

Sr = 1.7 % = 19.4 mg. mol./100 g. P = 0.14 % = 45 mg. mol./100 g.

After about 21 days, when the X-ray examination showed evidence of marked rickets, the animals were either sacrificed or transferred to the calcium rachitic diet for 3 to 4 days, and some of the tibiae removed for observations of *in vitro* calcification. The technique of Shipley *et al.* [1926] was used, artificial serum solutions having Ca $\times$ P product of 60 being employed as media in which slices of the growing ends of the bones were bathed at 37°. After 20 hours the bones were removed, stained with silver nitrate, cleared and mounted. In all cases our previous observations were confirmed. The strontium rachitic animals showed no deposition whilst the calcium rachitic group showed about 2 (++++) to 3 (++++) healing.¹ Both tibiae of the remaining animals were used for the determination of phosphatase activity using the technique outlined previously. The experiments described below do not include the preliminary investigations which were necessary for the standardisation of the technique.

In the first experiment 25 animals were placed on the calcium diet and 21 on the strontium diet. After 21 days the animals were killed. The tibiae of four animals in each group were employed for the study of *in vitro* calcification. Both

¹ The degree of calcification is indicated as follows: + trace; ++ broken thin line; +++ almost complete thin line across the provisional zone; ++++ complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone including the primary tongues of cartilage; 3 (++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis.

tibiae of the remaining animals were removed by the technique described, and used for subsequent determination of phosphatase activity at p_H of 9.5. The results of the experiment are presented in Table III.

Table III. *Comparative phosphatase activities at p_H 9.5 in the tibiae of animals suffering from strontium and calcium rickets.*

	Ca rickets 21	Sr rickets 17
No. of animals used for phosphatase determinations (two tibiae from each)		
Mean initial weight in g.	36.3 $\pm$ 0.7	45.7 $\pm$ 0.8
Mean final weight in g.	54.9 $\pm$ 1.4	50.6 $\pm$ 1.1
Mean phosphatase activity (mg. P/hour/bone)	2.74 $\pm$ 0.07	2.66 $\pm$ 0.07
Diff.		0.08
P.E. _{diff.}		0.099
Mean phosphatase activity $\times$ 100		
Mean final weight	5.0	5.25
Calcification <i>in vitro</i> at Ca $\times$ P = 60	2 (+ + + +) 3 (+ + + +)	None

From Table III it will be observed that the amount of *in vitro* deposition is markedly less in each of the strontium-fed groups than in the control group. At a Ca $\times$ P product of 60 sections of the bones of the strontium-fed groups showed no deposition. In contrast to these findings the tibiae of the control group showed a heavy line involving the primary and secondary tongues of cartilage.

The mean phosphatase activity of the calcium group is 2.74 as compared with 2.66 in the strontium group. The difference of 0.08 is so small that it could not explain the marked differences observed in *in vitro* calcification studies. Furthermore, this difference is not statistically significant (see Table IV). It can therefore be safely concluded that the phosphatase contents of corresponding anatomical portions of bone are the same in both the above groups.

In the second experiment 40 animals were divided into three groups. Group A consisted of 16 animals and received the calcium rachitic diet; group B consisted of 13 animals and received the strontium rachitic diet; group C consisted of 11 animals which had also received the strontium rachitic diet at first. After 21 days 7 animals of Group A and all of Group B were sacrificed. The tibiae of

Table IV. *Comparative phosphatase activities at p_H 8.8 in the tibiae of animals suffering from strontium and calcium rickets.*

	Group A Ca rickets 7	Group B Sr rickets 10	Group C Sr rickets + 4 days of Ca rachitic diet 5
No. of animals used for phosphatase determinations (two tibiae from each)			
Mean initial weight in g.	39.0 $\pm$ 1.1	44.3 $\pm$ 0.9	33.4 $\pm$ 1.6
Mean final weight in g.	52.6 $\pm$ 1.4	49.2 $\pm$ 1.4	35.6 $\pm$ 1.8
Mean phosphatase activity (mg. P/hour/bone)	1.57 $\pm$ 0.03	1.67 $\pm$ 0.06	1.27 $\pm$ 0.05
Diff.		0.1	0.40
P.E. _{diff.}		0.0671	0.0781
Mean phosphatase activity $\times$ 100			
Mean final weight	3.0	3.4	3.57
Calcification <i>in vitro</i> at Ca $\times$ P = 60	2 (+ + + +) 3 (+ + + +)	None	2 (+ + + +) 3 (+ + + +)

3 animals in each group were removed for observation of *in vitro* calcification, the rest of the tibiae being used for determination of phosphatase activity at p_H 8.8. Group C was transferred after 21 days to the calcium rachitic diet. On each of the subsequent 3 days two animals were killed, and the tibiae used for observation of *in vitro* calcification. At the end of the third day the tibiae were similar in *in vitro* calcifying power to those of the control rats. The following day the remaining five animals of Group C were killed and the tibiae used for the determination of phosphatase activity at p_H 8.8. The results of this experiment are tabulated in Table IV.

From Table IV it can be seen that the mean phosphatase values of the control group and the strontium-fed group are again close to each other. In contrast to the previous experiment the strontium group here has somewhat higher values. At any rate, this difference is without significance when examined statistically. The mean phosphatase values of the strontium-fed group which had been transferred to the calcium rachitic diet appear to be significantly lower than either one of the other two groups. However, if the smaller size of the animals in this group is taken into consideration, the values are near those of the other two groups. Certainly, there is no increase of phosphatase activity in the last group as one might expect if this enzyme were involved in the restoration of *in vitro* calcifying power. It seems evident, therefore, from these experiments that the phosphatase activity of the bone is not involved in the markedly reduced calcifying ability of the bone cells in animals suffering from rickets due to strontium.

SUMMARY.

1. Comparative determinations of the phosphatase activities of bones obtained from rats with strontium and those with calcium rickets were made. It was found that there was no significant difference in the phosphatase activities in similar anatomical portions of the bones from the two groups of animals.

2. The phosphatase contents of the bones of animals which were transferred from a strontium to a calcium rickets-producing diet were also determined. The mean phosphatase value of this group was somewhat less than that of the previous two groups, but when adjustments are made for the smaller mean weight of this group the mean value approaches those of the other groups.

3. Strontium ions did not affect the hydrolysing activity of phosphatase even in concentrations as high as 80 mg. of Sr^{++} in 100 ml. of hydrolysing mixture.

4. From this evidence it is concluded that there is no relationship between the marked loss of *in vitro* calcifying power and phosphatase activity in the bones of animals suffering from strontium rickets.

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