

CCCXII. THE NATURE OF THE INJURY TO THE CALCIFYING MECHANISM IN RICKETS DUE TO STRONTIUM.

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WHEN young rats are fed on diets containing strontium carbonate they become rachitic. The bones of these animals are characterised by a markedly diminished calcifying mechanism [Sobel *et al.* 1934]. At present, the nature of the alteration in the bone cell causing this variation is quite obscure. In fact, the entire problem of the factors operating locally in the bone cell is still unsolved. Whilst there are a number of theories as to what constitutes the "local factor", the bone "phosphatase mechanism" is the only one supported by satisfactory experimental evidence [Robison, 1932; Robison and Rosenheim, 1934; Niven and Robison, 1934; Fell and Robison, 1934]. Even this enzyme is known not to be the only factor involved as was confirmed in our studies of strontium rickets [Sobel *et al.*, 1935]. A further study of strontium rickets is therefore of importance because of the information that may be revealed regarding the factors responsible for calcification at the site of deposition. The present experiments seem to point to a "competitive retardation" of Sr^{++} upon the action of a constituent of the bone cell whose concentration is a factor in calcification. This experimental evidence is discussed below.

Referring to the results in our recent paper [Sobel *et al.*, 1934] the question arose whether or not the destruction of the calcifying mechanism is complete in strontium rickets. This was studied by using artificial serum solutions having a $\text{Ca} \times \text{P}$ product higher than 60 for observations of *in vitro* calcification in the bones. In this manner it was shown that at a $\text{Ca} \times \text{P}$ product of 90 the amount of *in vitro* calcification corresponds to that obtained at a $\text{Ca} \times \text{P}$ product of 40 in the control group. This observation suggests that besides the concentration of Ca^{++} and PO_4^{\equiv} the concentration of at least one other factor is involved in bone formation. In strontium rickets this factor appears to be reduced and therefore the $[\text{Ca}^{++}]$ and $[\text{PO}_4^{\equiv}]$ must be increased to compensate.

However, another possible explanation suggested itself. Strontium ions might remove the available phosphate ions by forming a unionised complex. This has been regarded as the most likely mechanism responsible for the inhibitory effects of magnesium ions [Shelling *et al.*, 1928] and of protein [Shipley *et al.*, 1926] upon calcification *in vitro*. To determine whether or not this is so a study of the effect of Sr^{++} upon calcification *in vitro* was undertaken. It was first demonstrated that Sr^{++} inhibits calcification *in vitro*. This inhibition was then studied quantitatively using bone slices from animals with calcium rickets. The sections were incubated in artificial serum solutions with a $\text{Ca} \times \text{P}$ product of 60 ($\text{Ca} = 10 \text{ mg./100 ml.}$, $\text{P} = 6.0 \text{ mg./100 ml.}$) and varying amounts of Sr^{++} . 2.0–2.5 mg. of Sr^{++} in 100 ml. of solution were sufficient completely to

inhibit calcification *in vitro*, although a $\text{Ca} \times \text{P}$ product of 35 is sufficient in the absence of Sr^{++} for *in vitro* deposition. Even if it is assumed that Sr exists as a completely unionised phosphate where the Sr/PO_4 ratio is similar to that in SrHPO_4 , 2.5 mg. of Sr would remove only 0.88 mg. of P which would still leave an effective $\text{Ca} \times \text{P}$ product of 51 (10×5.1), quite ample for *in vitro* calcification. Thus, it may be seen that this inhibitory effect of Sr^{++} may be explained by assuming that Sr acts directly upon a constituent of the bone cell. In view of the fact that the *in vitro* deposition of Sr has been demonstrated [Robison and Rosenheim, 1934] a competitive behaviour between Sr^{++} and Ca^{++} for a factor residing in the bone may be readily imagined. Such an explanation also harmonises the inhibitory effect of Mg^{++} upon *in vitro* calcification with the reported deposition of Mg *in vitro* [Robison and Rosenheim, 1934].

Whilst the above explanation is very attractive, there may be two other explanations for the interference of Sr^{++} with calcification within the bone cell. (1) Formation of a soluble Sr-Ca phosphate complex. This would explain the phenomenon on a purely physico-chemical basis. (2) Impairment of a vital function of the bone cell due to the toxic action of Sr. In the first case normal calcification should be re-established as soon as the Sr^{++} is removed. Since the diffusion of Sr^{++} should be rapid in a solution free from Sr, the restoration of the calcifying mechanism should take place readily. In the second case the damage should be practically irreversible, since there is no reason to believe that vital function will be restored unless the damaged tissue is replaced. If the inhibition is caused by a competitive behaviour between Sr and Ca as outlined in the previous paragraph, then the damage to the calcifying mechanism should be reversible. The rate of such a change would depend upon the velocity at which the Sr complex breaks down. This process would in all likelihood be slower than mere diffusion as postulated in the first possibility.

The question of the reversibility of the injury to the calcifying mechanism in strontium rickets was therefore studied both *in vivo* and *in vitro* in experiments in which the strontium-treated bone cells were subsequently bathed in fluids that did not contain Sr^{++} .

To demonstrate this reversibility *in vivo*, animals which had at first developed strontium rickets were transferred to the calcium rachitic diet and daily observations were made of *in vitro* calcification. At the end of 3 days the *in vitro* response was similar to that of the control group. Thus, a restoration of the calcifying mechanism was readily observed by *in vivo* methods. It was of interest to note that after 2 weeks on the calcium rachitic diet the average weight of the animals attained that of the controls. Moreover, *in vivo* healing could be readily observed in 7 days if vitamin D accompanied the calcium rachitic diet.

To determine whether the restoration of the calcifying mechanism occurred *in vitro*, slices of bones from animals with strontium rickets were incubated for 72 hours instead of the customary 20 hours at a $\text{Ca} \times \text{P}$ product of 60. After 72 hours there was a marked increase in the amount of deposition in the strontium group when compared with the degree of calcification in 20 hours. These results are remarkable since it is known that the calcifying mechanism in the usual type of rachitic section disappears in about 20–24 hours when immersed in an artificial serum solution having too low a $\text{Ca} \times \text{P}$ product for new deposition [Robison and Rosenheim, 1934]. This loss of the calcifying mechanism was confirmed by the present authors. Moreover, the control sections in the above experiment showed maximum amount of *in vitro* calcification in about 20 to 24 hours. Apparently, in the strontium group there is a gradual restoration of the calcifying mechanism whilst the reverse process takes place

in the control group during the same time. According to the above conception, it can be postulated that Sr in some manner prevents destruction of the calcifying mechanism. To explain this, one must assume the existence of a stable Sr complex with the same factor whose loss is a reason for the gradual destruction of the calcifying mechanism in the control group. If this complex undergoes the slightest dissociation then ultimately there will be an almost complete release of this factor in the free state, since the Sr will continue to diffuse out of the cell in a medium in which the concentration of Sr^{++} is comparatively low. In such fashion the gradual increase of calcification can be explained. This explanation would be in harmony with the theory of a competitive action of Sr with Ca for a factor whose concentration plays a part in calcification.

It can readily be seen that the other two explanations offered for the interference of strontium with the calcifying mechanism cannot hold. If the injury were a purely physico-chemical mechanism, then a gradual decrease in calcification should take place in the strontium group during a prolonged period of time just as it does in the control group, since it is likely that Sr could not preserve the bone-forming mechanism unless it were in combination with it. On the other hand, if the injury were inflicted upon the vital action of the cell then simple removal of Sr would not cause a restoration of the vitality of the cell in a purely inorganic solution which can hardly allow for the building up of organic tissue.

EXPERIMENTAL.

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 of age at which time they were placed on the stock diet. At 3 to 4 weeks of age administration of the experimental diets was begun. These were modifications of the Steenbock-Black [1925] rickets-producing diet which consisted of a basal regimen with addition of Ca or Sr as follows:

| | Basal diet 1 B ₁ | Basal diet 2 B ₂ |
|---|--------------------------------|--------------------------------|
| Cornmeal (Quaker Oats) | 71 g. | 70 g. |
| Wheat gluten | 20 g. | 16 g. |
| Brewer's yeast (Mead's) | 5 g. | 10 g. |
| NaCl | 1 g. | 1 g. |
| | P=0.23 g. =7.4 mg. mol. | P=0.15 g. =4.8 mg. mol. |
| Ca rachitic diet—Basal diet 97 g. + 3 g. of CaCO_3 Ca = 1.2% = 30.0 mg. mol./100 g. | | |
| Sr rachitic diet—Basal diet 97 g. + 3 g. SrCO_3 Sr = 1.7% = 19.4 mg. mol./100 g. | | |

In the first experiments diet B₁ was used. Later on, when a favourable effect of diet B₂ was noted in the growth of the calcium rachitic group, this diet was used in producing strontium rickets. In the latter case, both the growth curve and the *in vitro* response of the bones were similar to those on diet B₁.

The animals were X-rayed regularly once a week for the duration of the experiment. Plate VI, figs. 1 and 2 are typical roentgenograms of a young rat suffering from rickets due to strontium and of one suffering from rickets due to calcium. It is to be noted that at the border zone of the metaphysis and diaphysis in the tibiae of the strontium-fed animal a double line is present. This double line seems to be characteristic of strontium rickets and persists even 2 weeks after transferring the strontium fed group to the calcium rachitic diet, which is as far as such a change has been followed by the authors. Histologically, the bone

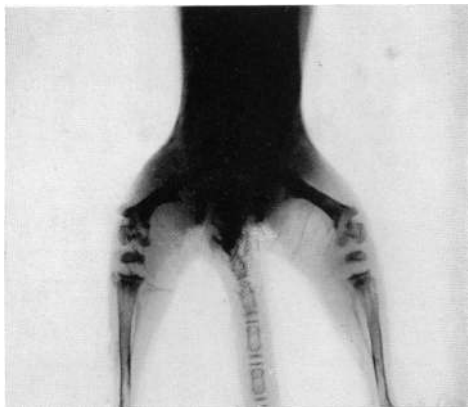


Fig. 1. Roentgenogram of the rear part of a rat suffering from rickets due to strontium.

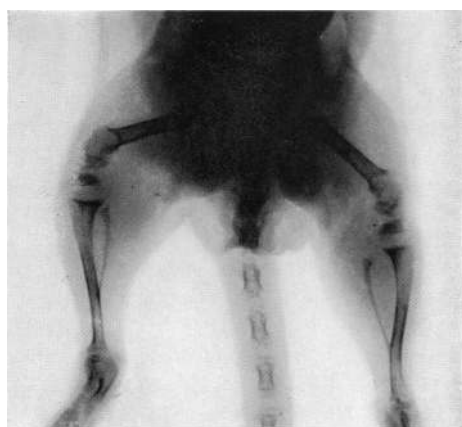


Fig. 2. Roentgenogram of the rear part of a rat with control rickets.

picture is essentially that described by Shipley *et al.* [1922]. There is persistence of the proliferating cartilage with increased osteoid tissue, the epiphysodiaphyseal junction is irregular and resorption of the bones is checked.

The percentage bone ash in general had a tendency to decrease with prolonged experimental period and increase with the initial age of the experimental animals. The average values for the bone ash percentage of the fat-free femora from the strontium groups varied from 28% to 35% in different experiments after 21 days on the experimental diet. Most of these were between 31 and 34%. There was no significant difference in the bone ash values between the animals on diet B₁ and diet B₂. In the calcium group the bone ash values of the animals on B₁ diet ranged around 29% whilst those for the B₂ group ranged around 34%.

The amount of growth was negligible in the strontium group during a 3-week period on the diet. During a similar period the weight gain of the calcium group on diet B₁ averaged 15–25 g. and that of those on diet B₂ averaged 40–50 g.

It was also observed that animals which were transferred from a 14-day strontium regimen to a subsequent 14-day calcium diet caught up in weight to those on the calcium diet for 28 days.

At the end of the experimental period the animals were sacrificed and the tibiae were removed for observations of *in vitro* calcification. The technique of Shipley *et al.* [1926] was used, artificial serum solutions having various Ca × P products being employed as media in which slices of the growing ends of the bones were bathed at 37°. After 20 or 72 hours the bones were removed, stained with silver nitrate, cleared and mounted.

Most of the experiments were reproduced 3 to 4 times. For the sake of brevity only typical experiments are described.

Calcification in vitro of the bones of animals suffering from rickets due to strontium.

The *in vitro* responses of bones of animals on diets B₁ and B₂ were observed at 14, 21 and 28 days on the experimental diet. Similar results were obtained for these various periods. At a Ca × P product of 10 × 6 the strontium group failed to show calcification.¹ At a Ca × P product of 15 × 6 the degree of *in vitro* calcification was about + to + + + +. This corresponds to the degree of calcification observed in the controls at Ca × P 10 × 4. The control group at Ca × P 15 × 6 showed 4 (+ + + +) healing. All the above observations were taken after a 20-hour period of incubation.

The inhibitory effect of Sr⁺⁺ upon calcification in vitro.

Sections of tibiae from animals suffering from rickets due to calcium were incubated in an artificial serum solution with Ca × P 10 × 6 and varying amounts of Sr⁺⁺ in the form of SrCl₂. 2.5 mg. of Sr⁺⁺ in 100 ml. of solution were sufficient completely to inhibit calcification in all cases. 2.0 mg. of Sr⁺⁺ were sufficient to inhibit in a number of cases.

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.

The reversibility of the injury to the calcifying mechanism in vivo.

Observations were made of the *in vitro* response of the bones of animals which were maintained for 2 weeks on strontium diet B_1 followed by 2 weeks on calcium diet B_1 . Identical observations were also made on a control group of animals kept on calcium diet B_1 for 4 weeks. The results were similar in both groups. At $Ca \times P 10 \times 6$ the response was 2 (++++) and at $Ca \times P 15 \times 6$ it was 4 (++++). Another group of animals was given daily doses of 33 Steenbock units of viosterol after they were transferred to the calcium diet from the strontium diet. After 7 days on the viosterol and calcium diet regimen, marked healing was observed *in vivo* in these animals.

In the next experiments animals on the strontium diet for 3 weeks were transferred to the calcium diet and daily observations were made of the *in vitro* response of the tibiae at $Ca \times P 10 \times 6$ as compared with controls. After 3 days on the diet the rats in the strontium group showed an *in vitro* response similar to that of controls.

The reversibility of the injury to the calcifying mechanism in vitro.

Slices of bones from animals with strontium rickets and with calcium rickets were incubated in artificial serum solutions having $Ca \times P 10 \times 6$. Observations were made at 20 and at 72 hours. At the end of 20 hours the strontium group showed either no calcification or only the faintest traces of it. In contrast to these findings the control group showed 2 (++++) to 3 (++++) healing. After 72 hours the strontium group averaged +++ to ++++ healing whilst the control group showed about the same amount of calcification in 72 hours as in 20 hours.

The loss of calcifying mechanism in vitro.

To confirm the experiments of Robison and Rosenheim [1934] sections of bones from the calcium rachitic group were incubated for 4, 7 and 24 hours in an artificial serum solution of $Ca \times P 10 \times 2$ which is too low a product to cause *in vitro* calcification. After this preliminary immersion they were placed in solutions with $Ca \times P 10 \times 6$ and 15×6 for 24 hours. No healing was observed after the 24-hour immersion. After 7 hours there was practically no response at $Ca \times P 60$ whilst at 90 the amount of response was + to ++++, which corresponds to that usually obtained at $Ca \times P 40$. After 4 hours of immersion there was a slight response at $Ca \times P 10 \times 6$ corresponding to that obtained at $Ca \times P 10 \times 3.5$, whilst at $Ca \times P 10 \times 9$ the response corresponded to that usually obtained at $Ca \times P 10 \times 5.0$ that is ++++ to 2 (++++).

SUMMARY.

1. Comparative observations of *in vitro* calcification of bones obtained from rats with strontium and with calcium rickets were made. It was found that there was a marked diminution, but not a complete destruction, of the calcifying power of the bones of animals suffering from strontium rickets.
2. The injury to the calcifying mechanism, in the bones of animals suffering from rickets due to Sr, was shown to be reversible both *in vivo* and *in vitro*. This was accomplished by bathing the bone cells in fluids which were free of strontium.

3. The inhibitory effect of Sr^{++} upon calcification *in vitro* was determined. A high degree of inhibition was obtained, which favours the explanation that Sr^{++} has a direct effect upon some constituent of the bone cell.

4. It is suggested that Sr combines with a factor whose concentration plays a part in calcification and thereby reduces the rate of bone formation.

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