CALCIFICATION

X. AN X-RAY DIFFRACTION STUDY OF CALCIFICATION IN VITRO IN RELATION TO COMPOSITION*

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PLATES 1 AND 2

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The precise nature of the material which deposits in the calcification of rachitic epiphyseal cartilage in vitro, as indicated by silver staining, has never been ascertained, although it has been used as a criterion of calcification for almost 3 decades (1-3). Much of our knowledge of the mechanism of calcification and of the local factors responsible for the site of mineralization rests on the assumption that this argyrophil substance is new calcification (1-5). This was based on the findings of early workers, who showed that the distribution of silver staining material in the cartilage matrix following calcification in vitro appears similar to that occurring in healing rachitic cartilage in vivo (2, 3). Silver staining is not specific for calcification; therefore, information on the nature of the silver staining deposit would be helpful in evaluating the soundness of the use of calcification in vitro in studies of the calcifying mechanism. The results of x-ray diffraction studies are presented in this paper. Chemical studies will be presented elsewhere.

EXPERIMENTAL

Young rats of an original Wistar strain, 23 to 25 days of age, kept on the Bills stock diet (6), were placed on a rickets-producing diet (7) for 21 days. At the end of this period the animals were sacrificed and the tibiae removed. Three longitudinal sections were cut from each tibia through the proximal epiphyseal cartilage, in a frontal plane 0.2 to 0.3 mm. thick.

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One section from each tibia was placed in a basal solution to serve as the control, and the remaining four sections from each rat were divided among four calcifying solutions. Each section was suspended by a glass hook in a 125 ml. Erlenmeyer flask which was completely filled with solution and tightly stoppered to prevent pH changes.\(^1\) The calcifying solutions contained calcium and inorganic phosphate (expressed as phosphorus) in a basal solution (A) containing 70 mM of NaCl, 5 mM of KCl, and 22 mM of NaHCO\(_3\) per liter. The initial pH was adjusted with CO\(_2\). The sections were incubated for 18 to 20 hours at 37° and pH 7.4. Changes of pH during incubation were usually within 0.05, and no more than 0.1 pH unit. In obtaining the final data, several successive experiments were employed. After incubation, the sections were dipped briefly in distilled water and immediately blotted on filter paper with a smooth surface. The sections were then stored in a desiccator until used.

The dried specimens were placed over the collimating slit of a micro x-ray diffraction camera (8), while viewed with a microscope, so that the exact location of the section giving rise to each diffraction pattern was known. The x-ray beam was approximately 60 microns in diameter. Exposures of 6 to 8 hours, with copper K\(_\alpha\) radiation, were used.

**Results**

**X-Ray Diffraction Patterns of Calcification in Vitro**

Calcification *in vitro* could be observed in the epiphyseal cartilage of all bone sections which had been incubated in the calcifying solutions. The calcification was easily discerned as an opaque line through the epiphyseal cartilage when viewed with transmitted light.

X-ray diffraction patterns from areas within the zone of new calcification (*in vitro*) and from preexisting calcified bone showed an inorganic diffraction pattern of the apatite type, characteristic of normal bone. Fig. 1, A

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ca (mg. per cent)</th>
<th>P (mg. per cent)</th>
<th>Ca × P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>15</td>
<td>45</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
<td>3</td>
<td>45</td>
</tr>
</tbody>
</table>

\(^1\) It has been found by one of the authors (A. E. S.) that results of experiments by sterile technique are comparable to those obtained when chemically clean glassware and careful handling of the animals and solutions are employed, as in these experiments.
and B, shows x-ray diffraction patterns of two sections, with accompanying photomicrographs which indicate the areas on the section from which the diffraction patterns came. Some parts of the preexisting bone, particularly the trabeculae, showed orientation. No orientation could be detected in any of the diffraction patterns taken from the calcification in vitro.

Fig. 2 shows diffraction patterns taken from the epiphyseal cartilage of several sections which had been incubated in calcifying solutions containing different Ca:P ratios. All of these (except for the controls) show only the apatite type of inorganic pattern, although the Ca:P ratio of the solution varied from 15:3 to 3:15. Apatite could not be discerned in diffraction patterns from the epiphyseal cartilage of control sections incubated in the basal salt solution containing no Ca or P (Fig. 2, A1 and A2).

### Table I

<table>
<thead>
<tr>
<th>Calcifying solution*</th>
<th>Solid composition</th>
<th>Diffraction pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca:PO4</td>
<td>CO3:PO4</td>
</tr>
<tr>
<td>Ca per cent</td>
<td>P per cent</td>
<td>CaHP04.H2O, apatite</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>1.22</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>1.70</td>
</tr>
</tbody>
</table>

* Consisted of basal solution in addition to Ca and P; 37°; pH 7.3 to 7.4, for 48 hours.

### Conversion of Calcium Acid Phosphate to Apatite in Vitro

None of the x-ray diffraction patterns showed the presence of calcium acid phosphate, which has been reported as the first aggregate of calcification (9–11). Apparently CaHPO4 is not formed or is unstable under our conditions of calcification in vitro.

To test the possibility that CaHPO4 is converted to apatite, and thus cannot be detected, 15 mg. samples of CaHPO4·2H2O were incubated in 500 ml. of Solutions B and E for 48 hours. The initial pH was 7.3 to 7.4 and did not change by more than 0.2 pH unit during the incubation. Most of the supernatant fluid was removed by aspiration, and the remainder with the solid was filtered with suction upon a fritted glass filter. The solid was washed rapidly (suction) with a few small portions of distilled water, followed by acetone, and then air-dried. Analyses for calcium, phosphate, and carbonate were carried out by methods described elsewhere (12, 13), and Debye-Scherrer diffraction patterns were made. The results are shown in Table I.

In the solution containing 15 mg. per cent of Ca and 3 mg. per cent of
P, all of the solid was transformed to apatite, as revealed by x-ray diffraction and as calculated from the chemical composition. A considerable amount of carbonate was present in the precipitate. In the solution containing 3 mg. per cent of Ca and 15 mg. per cent of P, about one-third of the solid was transformed to apatite, as calculated from chemical composition. A small amount of carbonate was present in the precipitate. X-ray diffraction revealed the presence of both CaHPO₄·2H₂O and apatite.

DISCUSSION

The x-ray diffraction patterns show that the material which deposits in calcification in vitro is an apatite. Thus, a sounder basis for silver staining as an index of calcification in vitro is presented. The pattern found was identical with that of preformed bone of the shaft or epiphysis of the sections, except for orientation. However, orientation cannot be excluded until studies of very thin sections are undertaken.

Chemical analyses to be reported elsewhere (13) show that the composition of the calcification in vitro varies with that of the calcifying solution. The Ca:PO₄ ratio (molar) varied from 1.43 to 1.64, and the PO₄:CO₃ ratio (molar) varied from 3.70 to 8.35. Despite these differences in composition, all of the diffraction patterns are identical. A similar situation has been shown to exist for bone, for which, in spite of wide variations in composition, x-ray diffraction reveals only apatite (14-16). Precipitates of calcium phosphate in which the Ca:PO₄ ratio varied from 1.28 to 1.63 all showed one predominant x-ray diffraction pattern, namely apatite (17, 18). It may seem incongruous that substances with such great differences in composition should all give the same diffraction pattern. This may be due to isomorphous substitution, which apatites are believed to undergo to a considerable extent (19). On the other hand, the extremely small crystals of apatites or tertiary calcium phosphate are capable of adsorbing relatively large amounts of other substances (20-23). Such adsorbed material does not usually show up in a diffraction pattern (24); hence only the apatite pattern is seen. If this is the case, then the composition of the structural part of bone salt or apatite may be relatively constant, the differences in over-all composition being due to adsorbed materials.

CaHPO₄ has been suggested as the first aggregate formed in calcification (9-11), which subsequently changes to tertiary calcium phosphate. The correctness of this concept was implied in the composition studies of inorganic systems (20, 25, 26). Logan and Taylor (20) reported that, in their inorganic systems, conversion of calcium acid phosphate to a tertiary calcium phosphate was slower in solutions containing higher PO₄:CO₃ ratios. These conclusions were drawn from composition studies and not x-ray diffraction. Sobel, Rockenmacher, and Kramer (12) reported that
bones of rats kept on a high phosphate-low calcium diet contained excess phosphate which could be expressed empirically as CaHPO₄. Nevertheless, in an x-ray diffraction study of such bones, Hirschman, Sobel, Kramer, and Fankuchen (14) could not demonstrate the presence of CaHPO₄ as such. The studies of McLean et al. (5) and Sobel and Hanok (7) imply that the first aggregate of calcification in vitro is CaHPO₄. However, in the present investigation, no CaHPO₄ could be demonstrated in calcification in vitro. This does not exclude the possibility that CaHPO₄ is the first aggregate, since the experiments on the incubation of CaHPO₄ show that it is unstable under these conditions. The transformation to apatite takes place more rapidly in the high calcium solution than in the low calcium solution. Klement (27) demonstrated earlier the instability of CaHPO₄ in Tyrode’s solution. Because of their relatively large surface area, newly formed aggregates would change more quickly than fully formed crystals in a solution in which they are not stable. Therefore, if CaHPO₄ were deposited in calcification occurring in vitro, it would be transformed into apatite more rapidly than fully formed crystals added to the calcifying solution. Our results indicate that, if CaHPO₄ were originally present in calcification in vitro, it could have been transformed into apatite in situ. However, it must be made clear that the data in this paper do not contain any direct evidence for the existence of CaHPO₄.

Studies of x-ray diffraction patterns of the mineral deposited in the first few minutes, rather than those obtained after several hours, may throw further light on the nature of the first aggregate deposited. In such studies the rapid calcification method in vitro recently reported may be of value (28).

SUMMARY

1. X-ray diffraction patterns of the material which deposits in calcification in vitro show only the apatite lines, despite the wide variation in chemical composition. Preformed bone gives identical patterns, except for orientation in some areas.

2. X-ray diffraction patterns fail to reveal the presence of CaHPO₄ postulated as the first aggregate formed in calcification. This, however, does not exclude CaHPO₄ from consideration, since CaHPO₄ is converted to apatite when placed in solutions employed for calcification in vitro.

BIBLIOGRAPHY


EXPLANATION OF PLATES

PLATE 1

Fig. 1. A, x-ray diffraction patterns obtained after calcification in vitro in a solution of Ca × P = 5 × 9. 3 and 4 from areas of calcification in vitro; 1, 2, and 5 from precalcified bone. B, x-ray diffraction patterns obtained after calcification in vitro in a solution of Ca × P = 15 × 3. 3 and 4 from areas of calcification in vitro; 1 and 2 from precalcified bone.

PLATE 2

Fig. 2. X-ray diffraction patterns of calcification in vitro in the basal salt solution containing various amounts of calcium and phosphate. A1, A2, Ca 0, P 0; B1, B2, B3, Ca 3, P 15; C1, C2, C3, Ca 5, P 0; D1, D2, D3, Ca 0, P 5; E1, E2, E3, Ca 15, P 3 mg. per cent. Each x-ray diffraction pattern is from a separate section.