Calcification XXI. Detection of Nuclei of Crystallization in Rachitic Cartilage.* (24344)

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Attempts to characterize mineralization of preosseous cartilage indicate existence of 3 discrete phases, starting with formation of nuclei of crystallization, continuing with their growth, and ceasing upon attainment of a finite crystal size. Ability to distinguish between these phases is essential to further our understanding of the underlying mechanisms involved, and to avoid anomalous results. The critical step in this process is the formation of nuclei, since extra-cellular fluids appear to be supersaturated with respect to bone mineral (1). It must be understood that the existence of nuclei necessary for crystal growth represents a concept. It is the smallest particle on which crystal growth can take place, in a given supersaturated solution. Formation of such nuclei according to this concept is more difficult than subsequent crystal growth. The calcifiable matrix presumably mediates formation of nuclei for growth of bone crystals. Direct visualization, or other methods of differentiating calcifying systems containing nuclei are needed for the understanding of the primary steps of calcification.

Methods of differentiating rachitic bone sections which have been immersed in calcifying solution from fresh sections or controls immersed in the same solution without calcium and phosphate are described. With our existing knowledge the most probable interpretation of these differences is that nuclei of crystallization or micro-crystals have formed below the threshold of visibility of the silver stain, particularly since the same differences were found when sections were employed in which some calcification was detected by the silver stain. We will refer to rachitic bone sections exposed to the calcifying solution for 2 hours as "nucleated" sections. This does not exclude some other possible explanation for the differences found. The first method is use of magnesium combined with iodoacetate. Previous studies by DiStefano(2) and from this laboratory(3,4) have shown that tibial sections treated for 2 hours in calcifying solution form nuclei whose further growth is no longer inhibited by iodoacetate. As seen in Table I, iodoacetate in conjunction with magnesium ion inhibits calcification *in vitro,* before nuclei formation, far more decisively than the iodoacetate alone. There is very little or no inhibition by these ions after "nucleation." The synergistic behavior of magnesium and iodoacetate appears similar in principle to the cation-linked inhibition of cyanide and fluoride previously reported(5-7). The second method of differentiating between "nucleated" sections and the nuclei-forming mechanism in non-nucleated sections, was to shake rachitic sections in distilled water either for half an hour or 1 hour. Following such treatment, all fresh (non-nucleated) sections lost ability to calcify. The "nucleated" sections, *i.e.* treated for 2 or 4 hours in an *in vitro* calcifying medium of Ca x P = 50, retained the ability to calcify (Table II). Another means of nucleation, used in previous study(4), was the treatment of sections with calcium ion, followed by phosphate ion. Although this treatment is not a physiological one, chemical studies showed that there was a marked increase in *in vitro* mineralization subsequent to it. The third method of differentiating between "nucleated" sections and the nuclei-forming mechanism was to immerse rachitic sections for 10 or 30 minutes in distilled water that had been preheated and maintained at

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76-77°C in a constant-temperature bath. Following such treatment, fresh sections, even after 10 minutes, lost ability to calcify. The "nucleated" sections, previously treated for 2 hours in an *in vitro* calcfying medium of Ca x P = 50, retained ability to calcify even after 30 minutes at 76°C, on 18 hours' incubation in *in vitro* calcifying solution (with a Ca x P of 50), or a similar solution which contained in addition 3 mM iodoacetate and 1 mM magnesium ions/l (Table III). An interesting aspect of the "nucleated" sections was that calcification *in vitro* could be obtained in trace amounts at a Ca x P product of 10 and more definite amount at a Ca x P product of 20. The non-nucleated (fresh) sections from animals that never lost weight while on the rachitogenic diet showed a trace of *in vitro* calcification at a Ca x P product of 30 and a more definite amount at a Ca x P product of 40 and above. The degree of *in vitro* calcification in fresh, non-nucleated sections at a Ca x P product of 40 corresponded to the degree of *in vitro* calcification in "nucleated" sections at a Ca x P product of 20. Some of these data are presented in Table IV. During the course of these studies, Thomas, Connor, and Howard(8) reported that tibial sections which had been partially calcified at a high product, or which had mineralized in the animal during an interval of forced starvation accompanied by loss of weight, could continue to calcify *in vitro* at a lower product than that required to produce calcification in fresh rachitic sections. Staining produced by the silver line test was above the threshold of visibility. In our experiments the sections were

TABLE I. Effect of Iodoacetate with and without Magnesium on *In Vitro* Calcification of Hypertrophic Epiphyseal Cartilage, with and without Pretreatment in Calcifying Medium.

Conc. of iodoacetate in Ca (10 mg %) × P (5 mg %) = 50 calcifying medium	Degree of *in vitro* calcification* after 18 hr in Ca (10 mg %) × P (5 mg %) = 50 calcifying medium: Pretreated 2 hr in Ca (10 mg %) × P (5 mg %) = 50 calcifying medium†	Not pretreated
Magnesium-free	mean	
0 mM iodoacetate	2.0 (++++)	1.8 (++++)
1	1.3 (++++)	1.0 (++++)
3	1.3 (+++)	1.3 (+++)
5	1.5 (+++)	.9 (+++)
10	1.2 (+++)	.6 (++)
Containing 1 mM/l magnesium		
0 mM iodoacetate	1.5 (++++)	1.5 (++++)
1	1.5 (++++)	trace
3	1.3 (++++)	0 (0)
5	1.3 (+++)	0 (0)
10	1.0 (+++)	0 (0)

* Degree of calcification was graded as follows: No calcification, 0 (0); maximum calcification, 4 (++++). Length of line of silver deposited is designated by +'s and its thickness or width by the No. preceding the parentheses. Since width of deposit is rarely uniform, the No. preceding the parentheses is an avg obtained by assaying each quarter of the line (under 32× magnification) and taking the avg.

† After 2 hr of pretreatment, the silver stain test was 0 (0).

TABLE II. Effect of Shaking Nucleated and Non-nucleated Sections with Distilled Water for Half an Hour and One Hour, Followed by Calcification *In Vitro*.

Treatment	Degree of *in vitro* calcification* after 18 hr: Ca (10 mg %) × P (5 mg %) = 50
Pretreated in Ca (10 mg %) × P (5 mg %) = 50 calcifying medium to produce nuclei:	mean
After 2 hr pretreatment,† shaken ½ hr in distilled water	1.0 (+++)
Idem † " 1 " " " "	.8 (++)
After 4 hr pretreatment,‡ " 1 " " " "	1.6 (++++)
Not pretreated:	
Shaken ½ hr in distilled water	0 (0)
" 1 " " " "	0 (0)
Not shaken " " "	1.8 (++++)

* First footnote, Table I, contains description of the code.

† After 2 hr of pretreatment, silver stain test was 0 (0).

‡ After 4 hr of pretreatment, silver stain test was 1.0 (++++), but deposit was of a very light gray color.

TABLE III. Effect of Heating Nucleated and Non-nucleated Rachitic Tibial Sections at 76° to 77°C.

Treatment	Degree of *in vitro* calcification* in solutions containing: Ca (10 mg %) × P (5 mg %) = 50	Ca (10 mg %) × P (5 mg %) = 50, + 1 mM magnesium + 3 mM iodoacetate
	mean	
Pretreated in Ca (10 mg %) × P (5 mg %) = 50 calcifying medium for 2 hr to produce nuclei:		
Heated 10 min. at 76°-77°C	1.8 (++++)	2.0 (++++)
" 30 " " "	2.0 (++++)	2.0 (++++)
Not pretreated:		
Heated 10 min. at 76°-77°C	0 (0)	
Not heated	1.8 (++++)	0 (0)

* First footnote, Table I, contains description of code.

definitely rachitic and no silver stain could be detected in sections that were pretreated for 2 hours at a Ca x P product of 50. Thus it may be concluded that nuclei below the threshold of silver stain visibility undergo growth at a lower Ca x P product than that necessary for formation of these nuclei. The clinical significance of this phenomenon has been amply discussed by Thomas *et al.*(8). Of the foregoing methods of testing for "nucleation," the iodoacetate inhibition in the presence of magnesium appears to be the most sensitive. The processes of heating or shaking minimally "nucleated" sections in water for long periods of time may conceivably dissolve the nuclei. Calcification at a lower product, while it is, by definition, most typical of the presence of nuclei, in itself cannot be regarded as a specific test since this may be obtained in embryonic cartilage(9); following preliminary shaking with calcium chloride(10); following treatment with calcium, then phosphate ions(3,11); and following treatment with calcium-ATP mixtures (12). Therefore, it is important to establish the presence or absence of nuclei in interpreting minimum products of calcification when studying: the roles of enzymes(2,9); effect of loss of weight of rachitic animals(3); effect of administration of hormones; and influence of heat, cold, chemicals and radiation on *in vitro* calcification, for if nuclei are present, erroneous conclusions may be drawn. In addition, the influence of injections *in vivo* of various substances or shifts in diet of experimental animals may produce nuclei rather than changes in the mechanism of nuclei formation, which hitherto was referred to as the calcifying mechanism. These studies indicate the need for differentiating between factors responsible for nuclei formation and those responsible for growth of nuclei to crystals, and emphasize the importance of these phenomena in future investigations, as well as re-evaluating calcification *in vitro* studies of the past.

Experimental. Weaned Wistar strain rats were fed a rachitogenic diet for 3 to 4 weeks and weighed daily, starting the fifth day on

TABLE IV. Effect of Various Ca × P Products on *In Vitro* Calcification of Hypertrophic Epiphyseal Cartilage with and without Pretreatment for 2 Hours in Calcifying Solution at Ca (10 mg %) × P (5 mg %) = 50.

Calcifying solution	Degree of *in vitro* calcification* after 18 hr in calcifying medium: Pretreated†	Not pretreated
	mean	
Ca × P = 10 (Ca = 2 mg %, P = 5 mg %)	trace	0 (0)
" = 20 (4 , 5)	1.0 (++)	0 (0)
" = 30 (6 , 5)	1.2 (+++)	trace
" = 50 (10 , 5)	2.0 (++++)	1.7 (++++)

* First footnote, Table I, contains description of code.
† After 2 hr pretreatment, silver stain test was 0 (0).

the diet. Tibial sections from animals that had not lost weight while on the diet were prepared as previously described(13,14). If healing was observed, the bones were discarded. The composition of the calcifying solution, except for indicated amounts of calcium, phosphate, magnesium, and iodoacetate, was as follows: NaCl, 0.07M; KCl, 0.005 M; and $NaHCO_3$, 0.022M. Pretreatment of tibial sections in the *in vitro* calcifying medium was accomplished by placing each slice into a 50 ml Erlenmeyer flask filled with the solution at pH 7.3 $\pm$ 0.05. The flask was tightly stoppered and placed in a 37° incubator for either 2 or 4 hours. After incubation, the sections were removed singly, with forceps, and rinsed with distilled water. The iodoacetate solution was made from sodium salt which was purified by dissolving the salt in a minimum amount of anhydrous ethyl ether. The white precipitate was filtered, washed several times with ether, and dried in a vacuum desiccator. Purity of the salt was determined by converting a sample to the acid. The melting point of the resulting iodoacetic acid was 82°C. Magnesium ion solution was prepared either from pure sample of magnesium carbonate, dissolved in redistilled hydrochloric acid, brought to dryness and dissolved in distilled water, or directly from pure magnesium sulfate. In either case the stock solution contained 1 M magnesium ion/l.

Summary. Methods of differentiating rachitic tibial sections that do not contain nuclei of crystallization in the preosseous cartilage from those that have been exposed to the calcifying solution but are below the threshold of visibility of the silver stain test are presented. These exposed sections are regarded as "nucleated" because they exhibit properties similar to those where calcification is visible with the silver stain test. "Nucleated" sections calcified *in vitro* while the non-nucleated sections showed complete inhibition when subjected to the following: (a) incubation in a calcifying medium (Ca x P = 50) containing a mixture of iodoacetate and magnesium ions; (b) shaking in distilled water at room temperature for half an hour, followed by *in vitro* calcification at a Ca x P product of 50; and (c) heating in distilled water at 76-77°C for 10 minutes, followed by *in vitro* calcification at a Ca x P product of 50. Calcification *in vitro* of "nucleated" sections took place at a lower Ca x P product than that needed for *in vitro* calcification of fresh, nuclei-free sections. It is suggested that the absence of nuclei should be established in studies of the nuclei-forming mechanism.

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