Calcification. V. Influence of Fluoride and Cyanide Ions in the Presence and Absence of Magnesium.* (19194)

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Robison and Rosenheim(1) reported that calcification of rachitic bone cartilage in inorganic medium is inhibited by 10^{-4} to 10^{-5} M fluoride, 10^{-3} M cyanide, and 10^{-3} M iodoacetate. Subsequently Gutman, Warrick, and Gutman(2) repeated and confirmed these observations in seeking to interrelate mineralization with phosphorylative glycogenolysis. Other groups of workers, however, including McLean *et al.*(3), have not obtained a significant interference by these ions that is not readily overcome by adding small increments of inorganic phosphate. More recently Marks and Shorr(4) have reported iodoacetate to be inhibitory while cyanide was found ineffectual in concentrations as high as 0.01 M.

We repeated several of these experiments and were unable at first to block calcification *in vitro* with either fluoride or cyanide. Indeed, raising the fluoride concentration to 10^{-3} M had the perverse effect of increasing, rather than decreasing, the area of mineralization. It has now been found, as shown below, that the origin of these discrepancies apparently lies in the role played by magnesium ion, whose presence is critical for the manifestation of fluoride and cyanide inhibition of calcification.

Experimental. A detailed description of the technic of *in vitro* calcification is given elsewhere(5). Bone cartilage sections were sliced from the rear tibiae of rachitic Wistar rats and suspended in the calcifying solutions from glass hooks. The four slices obtained from each animal were distributed between the experimental and control solutions. All incubations were carried out without shaking at pH 7.3 and 36.5°C. In addition to the test ions specified in the body of this paper, the calcifying solutions contained the following salts:

Salts	Molarity
NaCl*	.07
KCl*	.005
$NaHCO_3$*	.022
$CaCl_2$	.0025 (10 mg % Ca)
$NaH_2PO_4 + Na_2HPO_4$	.00161 (5 mg % P)

* The NaCl, KCl, and $NaHCO_3$ comprise the basal components of the calcifying solution.

The bone cartilage sections were stained with $AgNO_3$ to reveal the new mineral deposit and examined microscopically under 60 x magnification. The degree of calcification is designated in the following manner(6):

0 (0) —No calcification
1 (1+)—Trace
1 (2+)—Broken thin line
1 (3+)—Almost complete thin line across the provisional zone
1 (4+)—Complete thin line across the provisional zone
2 (4+)—Heavy line across the provisional zone including the primary tongues of cartilage
3 (4+)—Heavy line across the provisional zone including the primary and secondary tongues of cartilage
4 (4+)—Practically complete calcification of the metaphysis

Results. Effect of fluoride on calcification. In the absence of added Mg^{++} (*cf.* Table I) Slices of cartilage from rachitic rat tibiae were separated into two groups. The members of the control group were incubated for 18 hours in calcifying solution containing 10 mg % Ca^{++} (0.0025 M) and 5 mg % P (0.00161 M phosphate), while those of the second batch were incubated under the same conditions in calcifying solution supplemented with 10^{-4} M NaF. A comparative microscopic examination of the silver-stained tissues failed to reveal a distinct inhibition by fluoride. The mineral deposit formed in its presence appeared to be somewhat more shallow and to have a coarser honeycombed structure than the control, but the difference was not impressive. We have indicated that tibial sections in the absence of added inhibitors undergo calcification in the first 4-7 hours of incu-

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TABLE I. Dual Effect of Fluoride in the Absence of Added Mg^{++}.

Preliminary treatment, temp 37°	Fluoride conc. in calcifying sol., moles/l	Incubation period, hr	Degree of calcification: Test tissue	Degree of calcification: Control*
	.0001	5	0 (0)–1 (2+)	1.5 (4+)
	,,	18	1.5 (4+)	1.5 (4+)
	.001	5	2–2.5 (4+)†	2 (4+)
	,,	18	3 (4+) †	2 (4+)
Sections suspended 2 hr in basal sol. containing 5 × 10^{-5} M $BeCl_2$; pH 7.3	,,	18	0 (0)	2 (4+)
Sections suspended 2 hr in HCl, pH 2.7	,,	18	0 (0)	2 (4+)

* Refers to degree of calcification of parallel untreated tibial slices in the absence of fluoride ion.

† Deposit has structure, but lacks the sharpness and depth of the control.

bation(5). It was therefore reasoned that if fluoride does indeed retard ossification, then incubating over a period of 18 hours may obscure this effect by permitting a relief of inhibition or by permitting the reduced calcifying potential to operate for a greater length of time than is nominally required for the limited degree of calcification of the control. The incubation period was accordingly reduced to 5 hours, whereupon marked inhibition by 10^{-4} M fluoride was noted.

Although greater inhibition was anticipated at a fluoride concentration of 10^{-3} M, such was not found to be the case. Cartilage tissue incubated for 18 hours in calcifying solution containing 10^{-3} M fluoride calcified over a greater area than the control, although the deposit lacked in some degree the fine honeycombed character of the control. The difference was apparent even after 5 hours. Close inspection of the incubation flasks and test tissues failed to reveal a deposit or precipitate anywhere but in the metaphysis of the rachitic bones. *The enhanced mineralization in the presence of fluoride appears not to be an artifact but to require an active calcifying system.* Thus tissues inactivated by prior treatment with 5 x 10^{-5} M $BeCl_2$ or dilute HCl did not yield a deposit on subsequent incubation in fluoride-supplemented calcifying solution. The phenomenon of increased calcification at higher fluoride levels may reflect the formation of a fluorophosphate deposit(7). Composition studies would be required to establish this point.

In the presence of Mg^{++} (*cf.* Table II).

TABLE II. Fluoride Inhibition in the Presence of Added Mg^{++}.

Mg^{++} conc., moles/l	Fluoride conc., moles/l	Incubation period, hr	Degree of calcification: Test tissue	Degree of calcification: Control*
.001	.0001	5	0 (0)	2 (4+)
,,	,,	18	0 (0)	2 (4+)
,,	.001	5	0 (0)	2 (4+)
,,	,,	18	0 (0)	2 (4+)
.0005	.0001	5	0 (0)	2 (4+)
,,	,,	18	1 (2+)–1.5 (4+)†	2 (4+)
,,	.001	5	0 (0)	2 (4+)
,,	,,	18	1 (1+)–1.5 (3+)†	2 (4+)

* Tissues incubated in calcifying solution devoid of Mg^{++} and F^-. See Table I for effect of fluoride on calcification, and Table III for effect of Mg^{++} on calcification.

† Deposit tenuous and poorly integrated, with little depth.

While the results from the 5-hour runs underscored the initial inhibitory nature of 10^{-4} M fluoride ion, our inability to demonstrate interference with prolonged incubations was in striking contrast to the published observations(1) whose authenticity could not be doubted. A comparison of our experimental procedure with that of Robison revealed several differences, all but one of which were tentatively eliminated after some consideration. For example, the rats used in his studies were maintained on a rachitogenic diet for 3-4 weeks(8), whereas our animals were sacrificed after 2-3 weeks. Gutman and coworkers (2), however, were able to confirm Robison's fluoride experiment using rats kept on a rachitogenic diet for less than 2 weeks. This

TABLE III. Influence of Mg^{++} on the Course of Calcification.

Mg^{++} conc., moles/l	Incubation period, hr	Degree of calcification Test tissue	Control*
.0005	5	2 (4+)	2 (4+)
,,	18	2.5 (4+)	2.5 (4+)
.001	5	1.5 (4+)†	2 (4+)
,,	18	2-(4+)	2 (4+)
.002	5	0 (0)	2 (4+)
,,	18	0 (0)	2 (4+)

* No Mg^{++} present in calcifying solution.
† Mineral deposit stains more poorly than the control, indicating greater inhibition than is apparent from the 1.5 (4+) designation.

point of difference, therefore, seemed not to be critical.

Our attention was ultimately centered on the contributory role of Mg^{++} to inhibition. Experiments were conducted in which rachitic cartilage slices were incubated in calcifying solution containing both 10^{-4} M fluoride and 1 x 10^{-3} M Mg^{++} (added as $MgSO_4$ or $MgCl_2$). Calcification failed to occur in 18 hours. Similar results were obtained when the concentration of fluoride was increased 10-fold, in marked distinction from the excessive calcification noted in the absence of added Mg^{++} (Table I). Control studies were also carried out in which the influence of Mg^{++} *per se* on the course of calcification was determined (Table III). Calcification was found to be blocked at a Mg^{++} concentration of 2 x 10^{-3} M, partially inhibited in 5 hours of incubation but not appreciably in 18 hours by 1 x 10^{-3} M Mg^{++}, and unaffected by 0.5 x 10^{-3} M Mg^{++}. These results are in essential agreement with the data of Shelling, Kramer, and Orent(9), who found no interference in 18 hours by 0.74 x 10^{-3} M Mg^{++} but complete inhibition by 1.48 x 10^{-3} M Mg^{++}.

It is clear from Tables I, II, and III and the discussion above that the degree of calcification obtained in the presence of both 10^{-3} M fluoride and 1 x 10^{-3} M Mg^{++} falls far short of the results obtained with either component alone. We also sought to determine whether small quantities of Mg^{++}, *e.g.* 0.5 x 10^{-3} M, which do not interfere with calcification, do nonetheless accentuate the inhibitory activity of fluoride ion. The data obtained (Table II) were gratifying. Strong inhibition was noted after 18 hours of incubation in calcifying solution containing 10^{-4} to 10^{-3} M fluoride and 0.5 x 10^{-3} M Mg^{++}, while complete inhibition was obtained in 5 hours, even at the 10^{-3} M fluoride level (compare with Table I).

Effect of cyanide on calcification. In agreement with Marks and Shorr(4), we were unable to detect inhibition of calcification by prolonged incubation with solutions containing 10^{-3} M KCN (*cf.* Table IV). Cartilage slices withdrawn and stained after 5 hours of treatment yielded somewhat variable results, but in general the degree of calcification relative to the control was not appreciably inhibited by 10^{-3} M cyanide. On supplementing the cyanide-containing calcifying solution with 10^{-3} M $MgSO_4$ or $MgCl_2$, however, a complete block of calcification was obtained with the shorter incubation period. Strong inhibition was observed after 18 hours.

Discussion. It is evident from the data presented above that *in the presence of magnesium*, both fluoride and cyanide ions are effective inhibitors of calcification *in vitro*. The hitherto unrecognized role of magnesium explains our previous inability to obtain inhibition with prolonged incubation in calcifying solutions containing either of the two anions but devoid of magnesium, and probably resolves the discrepancies reported by others.† While the original procedure of Shipley, Kramer, and Howland(10) called for 8 x 10^{-4} M magnesium sulfate in the artificial calcifying media, many of the later workers in the field, including the authors, have usually

TABLE IV. Mg^{++}-Dependence of Cyanide Inhibition.

Mg^{++} conc., moles/l	Cyanide conc., moles/l	Incubation period, hr	Degree of calcification Test tissue	Control*
0	.001	5	1.3(4+)	1.5(4+)
,,	,,	18	1.5(4+)	1.5(4+)
.001	.001	5	0(0)	2(4+)
,,	,,	18	1(1+)-1(3+)†	2(4+)

* Degree of calcification in the absence of added Mg^{++} and CN^-. See Table III for the effect of Mg^{++}.
† Subsurface deposit.

† The reader is referred to pp. 17 and 20 of reference 12 and pp. 199-200 of reference 4.

omitted magnesium, since calcification proceeds readily in its absence. This is clearly the case with Marks and Shorr(4), whose failure to detect cyanide inhibition may be attributed to the lack of magnesium in their incubation solution. On the other hand Robison and Rosenheim(1), and later Gutman, Warrick, and Gutman(2), did observe interference by cyanide and fluoride. But they apparently did not associate the inhibition phenomenon with the specific activity of magnesium, which was included in their solutions in physiological concentration (0.001 M).

While fluoride ion is a potent inhibitor of calcification in the presence of an adequate concentration of magnesium, it appears to enhance calcification *in vitro* when used at a level of 10^{-3} M in the absence of added magnesium. This phenomenon suggests that in the early stages of bone, and possibly tooth development, the incorporation of fluorine in the apatite lattice(7) may proceed best in the presence of minimal amounts of magnesium. A comprehensive study of the relationship between dietary and serum magnesium and the effect of fluorine on the *early* growth of teeth seems to be called for.

The known presence of small amounts of magnesium in the experimental bone cartilage slices probably accounts for the initial inhibitory action of 10^{-4} M fluoride in calcifying solutions devoid of magnesium. On more prolonged incubation, much of this magnesium diffuses out of the cartilage(11). The resultant decrease in cartilage magnesium may then account for the observed relief of inhibition.

Presumptive evidence for the action of fluorine as an enzyme poison is afforded by its block of calcification in concentrations which are too low to affect the solubility of bone salts. Although a complete spectrum of the enzyme content of cartilage is not available, the ostensible requirement of magnesium for the manifestation of fluoride inhibition of calcification may be tentatively correlated with the presence of enolase in rachitic rat tibiae(12). From their studies on the properties of crystalline enolase, Warburg and Christian(13) have concluded that its specific inhibition by fluoride involves the formation of an inactive protein—magnesium fluorophosphate complex. It is significant that the activity of the enzyme, at a given concentration of fluoride ion, decreases with increasing concentration of magnesium. This may explain the need for adding magnesium in order to effect a decisive fluoride block of calcification. Further investigation is under way to determine whether the parallelism between the inhibitory action of magnesium and fluoride ions on enolase activity and on calcification is fortuitous.

The mechanism of cyanide inhibition is less readily amenable to interpretation(12). The deficiency in cartilage of cytochrome oxidase (11,14,15) and of respiratory and oxidative enzymes in general(11,14,16) seems to preclude the specific activity of cyanide here as an oxidative poison. Work in progress may help to elucidate the role of magnesium as a mediator of the cyanide inhibition of calcification.

Summary. 1. The *initial* rate of calcification of rachitic cartilage sections in inorganic media is markedly reduced in the presence of 10^{-4} M fluoride ion. With more prolonged incubation there is a relief of inhibition unless magnesium is included in the calcifying solution. 10^{-3} M fluoride is similarly effective in preventing mineralization provided the solution contains an adequate amount of magnesium; in its absence, the initial interference by 10^{-3} M fluoride is rapidly obscured by a secondary effect, interpreted as incorporation of fluoride in the growing mineral deposit, which gives rise to an apparent increase in calcification over the control. 2. Cyanide ion blocks calcification in the presence, but not in the absence of magnesium.

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Non-Effect of Cortisone on Growing Bones of Mice, Guinea Pigs and Rabbits. (19195)

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When cortisone acetate is administered to growing rats, a zone of increased density appears at the ends of the long bones and at the costochondral junctions(1). Such a metaphyseal area of increased density is composed of spicules of calcified cartilagenous matrix encased in bone. The pathogenesis of this change has been interpreted to be due to a decrease in normal osteolytic sequences(1). It seemed appropriate, therefore, to examine the skeletal tissues of several other species to which cortisone acetate* had been administered in order to determine whether changes similar to those encountered in the rat might be observed.

Experimental Mice. Weanling and full grown mice were given cortisone acetate in daily doses ranging from 10 to 75 mg per kilo. Animals were sacrificed from the 3rd up to the 23rd day. X-rays were taken of the upper ends of the tibiae; sections of these bones were studied microscopically. Aside from a retardation of growth in the young animals receiving 50 and 75 mg cortisone per kilo per day no changes were found in the metaphyses.

Guinea pigs. Guinea pigs weighing 180 to 200 g at the beginning of the experiment were given 5, 12.5, 25 and 50 mg cortisone acetate per kilo per day; animals were sacrificed at intervals from the 12th to the 37th day of treatment. X-rays and microscopic sections of the ribs and upper ends of the tibiae showed no changes save a retardation of growth in the animals receiving 50 mg cortisone per day.

Rabbits. Young rabbits, weighing 800 to 1000 g were given 5, 10, 25, 40, and 80 mg cortisone acetate per kilo per day and were examined at intervals after the 12th to the 37th day of such treatment. X-rays and sections of the ribs showed no changes save retardation in growth in the animals receiving 80 mg cortisone per day.

Discussion. Changes which may be produced in the rat's skeleton when appropriate doses of cortisone acetate (40-50 mg per kilo per day) are administered have been interpreted as being due to a failure of normal resorptive processes(1). In this they resemble bony alterations which may be encountered in the same species as a result of the influence of large amounts of estrogen. Under such circumstances normal osteolytic sequences appear to be inhibited(2,3). Since, in contrast to the rat, the effects of estrogen on the skeletal tissues of the mouse are so different and so striking, *i.e.* a tremendous stimulation of endosteal activity(4), a study of this species was approached with great interest. The results of cortisone administration, however,

* The cortisone acetate (Cortone) was kindly supplied by Merck and Co., Rahway, N. J.