Calcification IX. Influence of Alkaline Earths on Survival of the Calcifying Mechanism.*† (19991)

HARRY GOLDENBERG AND ALBERT EDWARD SOBEL.

From the Department of Biochemistry, Jewish Hospital of Brooklyn, Brooklyn, N. Y.

The purpose of this investigation was to study the influence of divalent cations on the survival of the mechanism responsible for calcification *in vitro* of hypertrophic epiphyseal cartilage. This was undertaken as an extension of our recent studies which indicate the existence of a constituent (or system) responsible for the disposition of cations(1-4). That some cations may influence the survival of the calcifying mechanism was already indicated in our earlier studies of strontium rickets, in which it was stated that "it can be postulated that strontium in some manner prevents the destruction of the calcifying mechanism"(5). Such a possibility was also indicated from recent studies where, by means of cations, the reversible inactivation of calcification *in vitro* was demonstrated(1,4).

In the present study calcification *in vitro* was determined following incubation in basal salt solution containing alkaline earth and other divalent cations. The results were compared to those obtained following incubation in basal salt only, in which there is a progressive decrease of the calcifying mechanism(6.7).

Experimental. The bone cartilage sections were sliced from the tibiae of Wistar rats that had been placed at the age of 22-24 days on a rachitogenic diet(4) for 14-21 days. To insure equal exposure of both sides of the slices, they were suspended in the test solutions in 25 ml Erlenmeyer flasks from glass hooks attached to lightly paraffined cork stoppers. The stopper was kept in place by a rubber band (ca. 3 mm wide to prevent rolling) run along a groove on the upper side and doubled under the flask back to the groove. In each experiment, at least one of the four slices obtained from each animal was used as a calcification control, and a second as a basal salt solution control. The degree of calcification of the silver stained sections was evaluated with a binocular microscope under 60x magnification, and graded according to the scale described previously(8), and illustrated in the Transactions of the Josiah Macy Foundation(9). The basal salt and calcifying solutions were prepared from stock solutions as described before(2,4), with the exception that dilutions were made with carbonated distilled water rather than with water followed by CO_2 treatment. Such solutions, initially below pH 7, were adjusted to pH 7.3 by swirling vigorously in a wide mouth Erlenmeyer flask. After basal treatment the sections were rinsed with distilled water and transferred to calcifying solution. All incubations were carried out without shaking at pH 7.30 $\pm$ 0.05 in a thermostatically controlled room whose temperature was set at 36.5 $\pm$ 0.5°C. *Basal Sol.* 70 mM NaCl, 5 mM KCl, and 22 mM $NaHCO_3$/liter. *Calcifying Sol.* Basal components plus 10 mg % Ca^{++} plus 5 mg % P as inorganic phosphate; Mg^{++} absent unless otherwise indicated.

Results and discussion. As seen in Table I, the loss of calcifiability of bone cartilage was markedly retarded by small amounts of calcium. Essentially similar results were obtained with strontium, although the mineral deposits were often not as well delineated as with calcium. The protective action of these two ions persisted for about 11-12 hours, whereas the basal salt controls underwent major loss of calcifiability in 7-8 hours and complete inactivation in 12 hours under our experimental conditions. For short periods of preliminary treatment the differences could be demonstrated when the subsequent periods of calcification were also short, generally of 4-6 hours duration, while the presence of the calcifying mechanism in sections soaked for a longer time could be better demonstrated

* Aided by grants from the National Institute for Dental Research of the National Institutes of Health, U. S. Public Health Service, and the Damon Runyon Memorial Fund for Cancer Research.

† Presented before the XIIth International Congress of Pure and Applied Chemistry, Sept., 1951.

TABLE I. Survival of Calcifying Mechanism in Basal Salt Solution Containing Divalent Cations. pH 7.3, 36.5°C.

Preliminary basal treatment: Added components*	Conc., mM/l	Incubation time, hr	Subsequent period of calcification, hr	Degree of calcification—previously soaked in: Basal + added comp.	Basal	Control
Ca^{++}	2.5	3	4	1.7(++++)	1(++)	2.3(++++)
		4	11	2(++++)	1.5(++++)	2.5(++++)
		7	4	1.9(++++)	0(0)	2.2(++++)
		7	5	2(++++)	1(+)	2(++++)
		13	12	0(0)	0(0)	2(++++)
		14	24	1.5(+)	0(0)	2.5(++++)
	0.625	8	13	2(++++)	1(++)	2(++++)
		11	22	1.5(++++)	1(++)	2.3(++++)
Sr^{++}	1.14	3	4	1.5(+++)	1(++)	2.3(++++)
		7	5	1.5(++++)	1(+)	1.7(++++)
		7	12	1.8(++++)	1.2(++)	2(++++)
		18	18	0(0)	0(0)	2(++++)
	0.285	8	13	1.5(++++)	1(++)	1.8(++++)
		11	22	1.2(+++)	1(++)	2.3(++++)
Mg^{++}	1	7	12	1(+)	1(+)	2(++++)
		7	12	1.6(++)	1.5(++)	3.3(++++)
		9	14	1(+)	1(+)	1.5(++++)
	4	4	4	0(0)	0(0)	1.5(++++)
		4	16	1.2(+++)	1(+++)	1.5(++++)
		6	13	1(+++)	1(++)	2(++++)
	10	7	12	1(+)	1(+)	2(++++)
		7	12	1.9(+++)	1.5(++)	3.3(++++)
Ba^{++}	1	2	6	1(+)	1(++)	2(++++)
		6	16	1(++)	1.1(+++)	2(++++)
		9	11	0(0)	0(0)	2(++++)
	5.0	3	19	1.4(+++)	1.1(+++)	2(++++)
		4	5	0(0)	0(0)	2(++++)
		6	17	1.8(+++)	1.6(+++)	2(++++)
		7	14	0(0)†	1(++)	2(++++)
		8	14	0(0)	0(0)	2(++++)
Be^{++}	0.05	2	16	0(0)	1.5(++++)	
Be^{++} + $PO_4^{\equiv}$	0.05 + 1.61	2	14	1.3(+++)‡	2(++++)	
Be^{++} + Mn^{++}	0.05 + 1	2	16	0(0)	1.5(++++)	
Mn^{++}	1	2	14	0(0)	2(++++)	
		1	18	1.2(++)	1.5(++++)	
Co^{++}	1	2	14	0(0)	2(++++)	
		1	18	1.4(++)	1.5(++++)	
Ni^{++}	1	1	18	1.1(++)	1.5(++++)	

* Mn^{++} and Mg^{++} were added as the sulfates, Co^{++} as the acetate, and the other cations as the chlorides. Phosphate was added as the sodium salt.

† A few flocculent white particles were noted on the stems of the glass hooks.

‡ Subsurface deposit.

§ Untreated section from same animal placed immediately in calcifying solution.

by more prolonged incubation in calcifying solution.

Of the other ions studied (Table I), barium had no apparent influence, in spite of the fact that it is a strong inhibitor when placed in calcifying media (unpublished experiments). Magnesium, which is a mild inhibitor of calcification(2-4,10), either had no effect on survival in basal solution or exerted trace protection. It did not interfere with the protective action of calcium when added to the basal medium, but did retard calcification when included in the subsequent mineralizing step (Table II). All of the other ions studied

TABLE II. Survival of Calcifying Mechanism When Tested in the Presence of Magnesium Ion. pH 7.3, 36.5°C.

Preliminary treatment,* 7 hr	Conc. of Mg^{++} in calcifying solution, mM/l	Degree of calcification, 12 hr
Basal	0	1(++)
+ Ca^{++}	0	1.9(++++)
+ Ca^{++} + Mg^{++}	0	2.2(++++)
+ Ca^{++}	1	1(+)
+ Sr^{++}	0	1.7(++++)
+ Sr^{++}	1	0(0)

* $(Ca^{++}) = (Sr^{++}) = (Mg^{++}) = 1$ mM.

were inhibitory, as shown by the fact that after 1-2 hours of immersion in basal solution containing 0.05 mM beryllium or 1 mM manganese, cobalt, or nickel, subsequent calcification did not occur.

When phosphate was present with beryllium ion in basal solution, only partial inhibition of calcification resulted. This finding indicates that studies of the inhibitory effect of beryllium when placed in a calcifying medium containing inorganic phosphate should be reevaluated(11-13), since inorganic phosphate interferes with the inhibitory effect of beryllium, probably by the formation of some unionized complex(4).

Actually, beryllium is a marked inhibitor of the inorganic mechanism as shown in these studies as well as previous studies of reversible inactivation(4). Further investigation is necessary to determine whether the block by beryllium is due to its binding of some tissue element, *e.g.*, chondroitin sulfate (4,14), or inactivation of an enzyme such as bone phosphatase(11-13,15). The ability of manganese to counter the beryllium inhibition of phosphatase(15) could not be put to a successful experimental test to resolve the phosphatase question, since manganese *per se* is inhibitory in basal medium.

The marked influence of calcium and strontium ions on the survival of the calcifying mechanism suggests that these ions form a relatively stable compound with an essential ingredient of the calcifying mechanism. Such a suggestion is compatible with earlier studies which have indicated that combination of some local factor with calcium is an integral part of the calcifying mechanism. In this connection, strontium also has an interesting history. It has already been shown that this cation is capable of yielding mineralized deposits from inorganic solutions(6,7), and evidence was presented that this ion blocks calcification by competing with calcium for some ingredient in the matrix essential for the calcifying mechanism. From the present evidence one might suggest a compound related to chondroitin sulfate(4,14), or adenosine triphosphate(16). This explanation would require that the dissociation constant of the postulated complex be small, in view of the low concentrations of calcium and strontium which retard disappearance of the calcifying mechanism. An alternate possibility is that strontium and calcium ions suppress a component or system which destroys or inactivates the calcifying mechanism.

Summary. 1. Rachitic bone cartilage slices which are suspended in basal medium at 36.5°C become slowly inactivated as shown by their subsequent inability to calcify. Major loss of calcifiability takes place in the first 7-8 hours with complete inactivation evident after about 12 hours. In the first 11 hours, small amounts of calcium or strontium exert a marked protective action against deterioration of the calcifying mechanism. Unlike calcium and strontium, comparable concentrations of magnesium and barium have no influence on the survival of the calcifying mechanism. 2. Beryllium in basal salt solution inactivates the calcifying mechanism, which can be prevented in part by inorganic phosphate. Manganese, cobalt, and nickel also inactivate the system when added to the basal salt solution. 3. Evidence to date favors the explanation that strontium and calcium ions protect the calcifying mechanism by forming a relatively stable compound with a component of the calcifying matrix essential for the mineralizing process, although an alternate explanation that these ions suppress a system responsible for inactivation cannot be excluded.

1. Sobel, A. E., Nobel, S., and Hanok, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 68.

2. Sobel, A. E., Goldenberg, H., and Hanok, A.,

PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 716.

3. Goldenberg, H., and Sobel, A. E., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 719.

4. Sobel, A. E., and Hanok, A., *J. Biol. Chem.*, 1952, v197, 669.

5. Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, 1935, v29, 2640.

6. Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, v28, 684.

7. Marks, P. A., and Shorr, E., *Science*, 1950, v112, 752.

8. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.*, 1935, v108, 395.

9. Sobel, A. E., *Trans. Macy Conference on Metabolic Interrelations*, 1950, v2, 113.

10. Shelling, D. H., Kramer, B., and Orent, E. R., *J. Biol. Chem.*, 1928, v77, 157.

11. Yu, T. F., and Gutman, A. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 481.

12. Gutman, A. B., and Yu, T. F., *Trans. Macy Conference on Metabolic Interrelations*, 1951, v3, 90.

13. Hiatt, H. H., and Shorr, E., *Trans. Macy Conference on Metabolic Interrelations*, 1951, v3, 105.

14. Rubin, P. S., and Howard, J. E., *Trans. Macy Conference on Metabolic Interrelations*, 1950, v2, 155.

15. Cochran, K. W., Zerwic, M. M., and DuBois, K. P., *J. Pharm. Exp. Therap.*, 1951, v102, 165.

16. Albaum, H. G., Hirshfeld, A., and Sobel, A. E., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 238.

Received September 17, 1952. P.S.E.B.M., 1952, v81.

Effect of Total-Body X-Irradiation on Relative Turnover of Nucleic Acid Phosphorus.* (19992)

ANITA H. PAYNE, LOLA S. KELLY, AND CECIL ENTENMAN.

From the Donner Laboratory of Medical Physics, University of California, Berkeley and Naval Radiological Defense Laboratory, San Francisco.

Supplee and Entenman(1) have observed that fasted adult female Sprague Dawley rats, previously subjected to large doses of total body X-irradiation, have larger livers than fasted non-irradiated control rats. This enlargement of the livers was found not to be due to edema or to the excessive accumulation of lipids. The maximum increase in liver mass occurred at approximately 24 hours following 2500r total body X-irradiation. Since the increase in liver size seems to be due to an actual increase in liver tissue, it was believed that this change might be reflected in the synthesis of cytoplasmic pentose nucleic acid (cPNA). It has been postulated by Casperson, Brachet, and Spiegelman(2-4), that the formation of PNA and protein are intimately related. Abrams(5) studied the effect of X-irradiation on the formation of desoxypentose nucleic acid (DNA), PNA, and protein in rat intestine. He observed that with 500r the incorporation of glycine-1-C^{14} into DNA and PNA purines is depressed, while there is no apparent change in the incorporation of glycine into proteins. This study might be interpreted to contradict the earlier observations of Casperson and Brachet. However, since the incorporation of the radioactive precursor was observed at only one time interval (*i.e.*, 48 hours), and since it is not known at which step between precursor and final product inhibition occurs, it cannot be concluded from Abrams' findings that there is no relationship between PNA and protein synthesis.

In the experiment reported in this paper we determined the incorporation of radioactive phosphorus (P^{32}) into rat liver DNA, cPNA, and nuclear PNA (nPNA) 24 hours post-irradiation, at which time the maximum effect of total body X-irradiation on liver weight was observed. We also determined the same nucleic acid fractions in mice exposed to 600r at 2½ hours post-irradiation.

Experimental. Twenty female *Sprague-Dawley* rats each weighing approximately 200 g were given a total dose of 2500r of 250 kv X-rays filtered through 0.5 mm Cu and 1 mm Al at an average dose rate of 22.7r/min. Food was removed from cages just prior to irradiation. Water was allowed *ad lib.*

* Partially supported by U. S. Atomic Energy Commission.