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Rapidity of Calcification *in vitro*.* (19461)

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Most experiments reported in the literature on the calcification of hypertrophic rachitic cartilage *in vitro* depend on 16 to 20 hour incubation periods. Generally, these have been performed with minimal concentrations of calcium and inorganic phosphate. We have observed that calcification can proceed with unexpected rapidity in organic phosphate ester, and that the characteristic line of calcification may be observed after as little as 5 minutes incubation.

Methods. Slices of epiphyseal cartilage were prepared from bones removed from young rats, which had been made rachitic by feeding them a modified Steenbock and Black diet No. 2965(1). They were shaken in a water bath at 37°C for varying periods of time in calcification medium, as described by Miller, Waldman, and McLean(2), then stained with 2% $AgNO_3$, and cleared with oil of isosafrol. Phosphate was supplied as inorganic phosphate, from a stock solution of 0.1 M phosphate buffer, pH 7.4, or as disodium monophenyl phosphate. Inorganic phosphate liberated from the organic ester was determined by the method of Gomori(3) after removal of the slices and precipitation of the contents of the flask with an equal volume of 10% CCl_3COOH. The calcification score used is that described by Sobel, Goldfarb, and Kramer(4).

Results. In a medium which contained only inorganic phosphate a wide line of calcification was noted in 5 of 6 slices after 8 hours incubation in the highest concentration of phosphate, 5.0 mM (Exp. 1, Table I). The density of the calcium phosphate deposit increased after 22 hours incubation. Little or no calcification was noted up to this time with 2.0 or 3.0 mM inorganic phosphate. In the presence of organic phosphate ester, however, all the slices showed calcification after only one hour incubation. At this time, the concentration of inorganic phosphate attained in the medium due to hydrolysis of disodium monophenyl phosphate was only 1.7 mM. As in the experiment with inorganic phosphate, the density of the bone salt deposit became greater with more prolonged incubation.

In order to determine how rapidly salt deposit can occur in organic phosphate ester, a second experiment was performed with a higher concentration of Ca^{++}, 2.0 mM. Flasks were removed from the incubator after various intervals of time, from 5 to 60 minutes. Beginning calcification was noted in 1 of 4 slices after only 5 minutes, when the inorganic phosphate content of the medium had reached

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TABLE I. Rate of Calcification of Rat Hypertrophic Rachitic Cartilage Slices in Inorganic and Organic Phosphate.

Exp. No.	Incubation	Ca^{++}, mM	Inorganic phosphate: Conc., mM	Inorganic phosphate: Calcification score	Disodium monophenyl phosphate: Conc., mM	Disodium monophenyl phosphate: Calcification score	Inorganic PO_4-liberated, mM
1	1 hr	1	2	6=0		3=4+, 1=2(4+), 2=3(4+)	1.7
			3	6=0			
			5	6=0			
	2	1	2	6=0		1=3+, 4=2(4+), 1=3(4+)	3
			3	4=0, 2=±			
			5	5=0			
	3	1	2	5=0	33	1=4+, 4=2(4+)	4.3
			3	7=0			
			5	5=0, 1=±			
	8	1	2	4=0, 1=±		3=2(4+), 2=3(4+)	7.2
			3	4=0, 1=±			
			5	3=2(4+), 1=3(4+), 1=±			
	22	1	2	2=0, 3=±		3=2(4+), 2=3(4+)	22.6
			3	2=0, 2=+, 1=2+			
			5	2=3+, 1=4+, 2=2(4+)			
2	5 min	2				3=0, 1=3+	.15
	10	2				3=4+, 1=3+, 1=±	.26
	15	2			33	5=2(4+)	.35
	30	2				1=4+, 4=2(4+)	.61
	45	2				2=2(4+), 3=4+	.70
	60	2				1=3+, 2=2(4+), 1=3(4+)	.86

See text for a discussion of the density of calcification.

a level of only 0.15 mM (Exp. 2). The line of calcification was very narrow and extremely faint, but was characteristic and in the normal position. After 10 minutes, 3 of 5 slices showed a narrow line of calcification extending across the provisional zone of calcification (4+), but still extremely faint. After 15 minutes incubation, all the slices exhibited a wide band of calcification which extended across the provisional zone of calcification and included the primary tongue of cartilage. Both the width of the band of calcification and the density of the calcium phosphate deposit increased with time of incubation. After one hour, when calcification was marked, the inorganic phosphate content of the medium had reached a level of only 0.86 mM.

Summary and conclusions. 1. In the presence of organic phosphate ester, calcification of hypertrophic rachitic cartilage slices occurs with great rapidity and at what appears to be an extremely low concentration of inorganic phosphate. The estimated concentration of inorganic phosphate ester is undoubtedly deceptive, because it does not indicate the concentration which prevails in the immediate vicinity of the slice and in the slice itself. This is probably extremely high because of degradation of organic phosphate ester by alkaline phosphatase in the bone slice, and the limited rate of diffusion of phosphate away from the slice. 2. Our results also indicate that in a very short time the outline of the final calcification pattern is formed, and the density of the bone salt deposit then increases with time.

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