THE EFFECT OF DYES ON THE CALCIFICATION OF HYPERTROPHIC RACHITIC CARTILAGE IN VITRO*

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(Received for publication, February 18, 1952)

It has been known for more than 50 years that chondroitin sulfate esters are widely distributed in all types of cartilage (1), and are probably responsible for the characteristic metachromatic staining by dyes such as toluidine blue (2–4). According to Hass and Garthwaite, epiphyseal cartilage, which is capable of calcifying, contains approximately 20 per cent of this compound (5).

Until recently, few investigations were directed at characterizing the fibrous matrix on which bone salt is deposited and clarifying the precise role of the matrix in the calcification process. Several years ago we performed some experiments designed to test the possibility that chondroitin sulfate esters might be involved in calcification. We approached this problem by studying the effect of dyes known to stain the intercellular matrix of hypertrophic cartilage in the hope that such dyes might influence calcification. Therefore, we investigated metachromatic dyes like toluidine blue, as well as other basic and acid dyes. Our experiments suggest that chondroitin sulfate may be essential for the calcification of hypertrophic rachitic cartilage.

Material and Methods

Rats (3 to 4 weeks old) weighing 50 to 60 gm. were placed on a modified (6) Steenbock-Black diet 2965 (7). The diet used contained 76 per cent corn meal, 20 per cent gluten, 1 per cent NaCl, and 3 per cent $CaCO_3$. A few experiments were performed with rabbits which were made rachitic by feeding them the McCollum-Simmonds diet 3143 (8) from the time they were 4 weeks old (300 to 350 gm.). This diet consists of 33 per cent wheat, 33 per cent corn meal, 15 per cent gelatin, 15 per cent wheat gluten, 1 per cent NaCl, and 3 per cent $CaCO_3$. After 3 to 4 weeks on this diet, when rickets were severe, the animals were killed by a blow on the neck. The bones of the limbs were removed with aseptic precautions. Slices less than 1 mm. thick were prepared with a razor from the upper end of the tibia, the lower end of the femur, and, in some instances, from the humerus.

The primary calcification medium used in all experiments was isotonic with serum and had the following composition: 825 cc. of 0.154 M NaCl, 35 cc. of 0.154 M KCl, 10 cc. of 0.1

* The experiments on which this paper is based were performed in 1946–47, and were supported by a grant from the National Institutes of Health, Public Health Service.

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M $MgCl_2$, 130 cc. of 0.154 M $NaHCO_3$. Suitable amounts of the calcification medium and 0.1 M sodium phosphate buffer, pH 7.4, were pipetted into 25 cc. Erlenmeyer flasks so that the final volume, after all additions including calcium had been made, was 10 cc. In a typical experiment, 0.6 cc. of phosphate buffer was added to 9.3 cc. of the calcification medium. One drop of 0.04 per cent phenol red was added. Rubber stoppers and small bent capillary tubes were inserted in the openings of the flasks which were then autoclaved at 18 pounds pressure for 15 minutes. When the flasks were cool, 0.1 M $CaCl_2$ was added, usually 0.1 cc., and the flasks were gassed aseptically, through sterile, cotton-plugged drawn-out tubes, with 95 per cent N_2–5 per cent CO_2 to pH 7.4.

In the experiments described by Robison and most subsequent investigators, the concentrations of calcium and inorganic phosphate used corresponded with those in serum, 8 to 10 mg. Ca^{++} and approximately 3.5 mg. P per 100 cc. These were usually limiting values. In some of our experiments, we deliberately used a different ratio of Ca^{++} and $PO_4^{=}$[1] to reduce precipitation, and a higher $Ca^{++} \times PO_4^{=}$ product than that required for minimal calcification, in order to rule out Ca^{++} or $PO_4^{=}$ concentration as the limiting factor. The concentrations used in each experiment are indicated in the tables.

Calcification in the presence of sodium glycerophosphate and disodium monophenyl phosphate was studied in some experiments. The substrates were used at a concentration of M/30 with respect to phosphorus. The calcium concentration was 0.01 mM/10 cc.

Three or four rats were used for each experiment, which permitted setting up as many as 20 flasks. Bone slices from each rat were distributed into all the flasks, so that the results would be comparable. Five or six slices were placed in each flask, and the flasks were shaken in a water bath at 37°C. for approximately 16 hours.

The inhibitory effect of the dyes on calcification was tested in two different ways. In some experiments, the inhibitor was present during the entire 16 hour period of incubation. In others, the dye was made up to pH 7.4, diluted to the desired final concentration with primary calcification medium without Ca^{++} or $PO_4^{=}$, and gassed as above. Rachitic cartilage slices were immersed in the solution for 1 hour at room temperature. The slices were then thoroughly rinsed with sterile physiological saline, and incubated in flasks containing Ca^{++} and $PO_4^{=}$ at 37°C. for the usual 16 hour period. Suitable controls were carried through the entire procedure.

At the end of the desired incubation period in calcification medium, the slices were washed several times with distilled water, then placed in shallow dishes containing 2 per cent $AgNO_3$. Satisfactory staining resulted after a few minutes exposure to bright sunlight. The slices were rinsed in about 50 cc. of distilled water; excess moisture was absorbed by filter paper, and the slices were dried in acetone for 5 minutes. They were cleared in oil of isosafrole, and examined under a dissecting microscope without mounting. Because the entire slice darkens within a few days after this procedure, they were examined shortly after clearing and discarded.

The calcification score used is that described by Sobel, Goldfarb, and Kramer (9): + trace; ++ broken thin line; +++ almost complete thin line across the provisional zone; ++++ complete thin line across the provisional zone; 2 (++++) heavy line across the provisional zone, including the primary tongue of cartilage; 3 (++++) heavy line across the provisional zone, including the primary and secondary tongues of cartilage; 4 (++++) practically complete calcification of the metaphysis. The results in our tables represent the average of readings on 5 or 6 slices. Non-specific precipitation was rarely seen. If the results were not clear-cut, the experiment was discarded.

When estimations of residual Ca^{++} and $PO_4^{=}$ concentrations were to be made, the slices were removed and the contents of the flask was precipitated by the addition of an equal volume of 10 per cent CCl_3COOH. This mixture was filtered through Whatman No. 42 filter

[1] Throughout this paper, the notation, $PO_4^{=}$, is used for the sake of convenience and should be understood to include $(HPO_4)^{=}$.

paper. Calcium was determined in the filtrate by the Clark and Collip (10) modification of the Kramer and Tisdall method (11), and phosphorus by Gomori's method (12).

TABLE I

Effect of Toluidine Blue and Crystal Violet on Calcification

Dye	Experiment No.	Concentration of dye	Time of exposure to dye	Average calcification score	Supplement
		M	*hrs.*		
Toluidine blue	1	0	0	<++++	
		10^{-4}	1	<+	
		10^{-4}	16	<++++	
Toluidine blue	2	0	0	2(++++)	
		10^{-4}	1	+	
		10^{-4}	16	+++	
Toluidine blue	3	0	0	2(++++)	
		10^{-4}	1	<+++	
		10^{-4}	1	>++	0.01 mM Ca^{++} in T.B.* solutions
		10^{-4}	1	+++	0.10 mM $PO_4^{=}$ in T.B.* solution
Toluidine blue	4	0	0	2(++++)	
		10^{-4}	1	<+	
		10^{-4}	1	<+	10^{-4} M orange G in T.B.
		10^{-4}	1	>+	3×10^{-3} M orange G solution
Toluidine blue	5	0	0	2(++++)	
		10^{-4}	1	+	
		10^{-4}	1	+	10^{-4} M orange G in calcifying
		10^{-4}	1	++++	3×10^{-3} M orange G solution
Crystal violet	6	0	0	3(++++)	
		10^{-4}	1	>+	
		10^{-4}	16	++++	
Crystal violet	7	0	0	2(++++)	
		10^{-4}	1	<+	
		10^{-4}	1	>+	3×10^{-3} M orange G in T.B. solution
		10^{-4}	1	>++++	3×10^{-3} M orange G in calcifying solution

* T.B. represents toluidine blue.

The calcifying solution in these experiments contains 0.01 mM Ca^{++} and 0.10 mM $PO_4^{=}$.

RESULTS

Table I shows typical results with two basic, metachromatic dyes, toluidine blue and crystal violet. If slices of hypertrophic rachitic cartilage are exposed for 1 hour to a 10^{-4} M solution of dye in the absence of Ca^{++} or $PO_4^{=}$, and then

washed and placed in calcifying medium for the usual 16 hour period, there is very marked inhibition of calcification. On the other hand, if the dyestuff is added directly to calcifying medium and is present during the entire 16 hour incubation period it has little or no effect at 10^{-4} M.

Since the only difference between these two experiments is that in the second case both Ca^{++} and $PO_4^{\equiv}$ are present, it appeared that either Ca^{++} or $PO_4^{\equiv}$, or both, prevented inhibition by the dyestuff. It was found that addition of either Ca^{++} or $PO_4^{\equiv}$ alone to 10^{-4} M toluidine blue during the pre-

TABLE II

Effect of Other Basic Dyes on Calcification

Dye	Concentration	Average calcification score
	M	
Acridine red	0	2(++++)
	10^{-3}	0
Hemotoxylin	0	>2(++++)
	10^{-3}	0
Janus green	0	2(++++)
	10^{-3}	0
Basic fuchsin	0	2(++++)
	10^{-3}	+
Safranin	0	2(++++)
	10^{-3}	+
Neutral red	0	2(++++)
	10^{-3}	<2(++++)

Rachitic cartilage slices were incubated at room temperature for 1 hour with dye, then washed and incubated for 16 hours in calcifying solution which contained 0.01 mM Ca^{++} and 0.06 mM $PO_4^{\equiv}$.

liminary 1 hour incubation period was without effect (Experiment 3). Apparently both ions are needed.

To determine whether the basic dye could be neutralized by an acid dye, tissue slices were exposed for 1 hour to a solution which contained either 10^{-4} M crystal violet or toluidine blue *plus* an acid dye, orange G. Under these conditions, orange G was without effect at 10^{-4} M or 3×10^{-3} M (Experiment 4). However, if the slices were first exposed to 10^{-4} M toluidine blue or crystal violet, and then washed and placed in calcifying solution which contained 3×10^{-3} M orange G, there was very little inhibitory action (Experiments 5 and 7). Apparently under these conditions the relatively small amount of dye fixed to the tissue can be leached out and neutralized by the orange G.

Typical results obtained after 1 hour exposure to other basic and acid dyes

are presented in Tables II and III. Most of the basic dyes studied inhibited strongly at 10^{-3} M, and some, irregularly, at 10^{-4} M. Neutral red was essen-

TABLE III

Effect of Acid Dyes on Calcification

Dye	Concentration	Average calcification score
	M	
Alizarin	0	2(++++)
	10^{-3}	0
	10^{-4}	<+++
Quinalizarin*‡	0	>2(++++)
	10^{-3}	0
Orange G‡	0	>2(++++)
	3×10^{-3}	>2(++++)
Orange G‡	0	++++
	10^{-2}	++
Titan yellow*	0	2(++++)
	10^{-3}	++
Titan yellow‡	0	(++++)
	10^{-3}	(++++)
Azocarmine	0	2(++++)
	10^{-3}	2(++++)
Acid fuchsin‡	0	2(++++)
	10^{-3}	2(++++)
Aniline blue	0	2(++++)
	10^{-3}	2(++++)
Aurantia	0	2(++++)
	10^{-3}	2(++++)

Rachitic cartilage slices were incubated at room temperature for 1 hour with dye, then washed and incubated for 16 hours in calcifying solution which contained 0.01 mM Ca^{++} and 0.06 mM $PO_4^{=}$ unless otherwise indicated.

* Incubated for 1 hour at pH 9.5

‡ Calcifying solution contained 0.01 mM Ca^{++} and 0.08 mM $PO_4^{=}$.

tially without effect. Few acid dyes were effective at these concentrations. However, alizarin and quinalizarin[2] inhibited to a very marked degree at

[2] Quinalizarin is insoluble at pH 7.4 at this concentration. It was therefore studied by incubating the slices for 1 hour in veronal-acetate buffer, pH 9.5. Control slices were carried through the entire procedure.

10^{-3} M. Because it is known that alizarin forms a lake with calcium, an attempt was made to prevent the alizarin inhibition by doubling the calcium concentration in the final calcifying solution. This procedure had no effect.

Titan yellow inhibited at 10^{-3} M at pH 9.5, but not at pH 7.4, Orange G inhibited at 10^{-2} M.

The effect of several dyes on calcification in the presence of organic phosphate esters is shown in Table IV. Corresponding results with inorganic phosphate are presented. In almost all cases, cartilage slices which did not calcify

TABLE IV

Effect of Dyes on Calcification in Inorganic and Organic Phosphate

Dye	Concentration	Inorganic PO_4	Average calcification score	
			Glycerophosphate	Phenyl phosphate*
	M			
Control	0	2(++++)	>2(++++)	
Toluidine blue	10^{-4}	>+	<2(++++)	
Hemotoxylin	10^{-4}	>++	>2(++++)	
Basic fuchsin	10^{-3}	<+++	++++	
Safranin	10^{-3}	<+++	++++	
Control	0	<3(++++)	2(++++)	>2(++++)
Alizarin	10^{-3}	0	++++	++++
Control	0	>2(++++)	<3(++++)	3(++++)
Quinalizarin‡	10^{-3}	0	>++	<++++
Control	0	>2(++++)		2(++++)
Titan yellow	10^{-3}	0		3(++++)

Rachitic cartilage slices were incubated at room temperature for 1 hour with dye, then washed and incubated for 16 hours in calcifying solution which contained 0.01 mM Ca^{++} and 0.06 mM inorganic $PO_4^{=}$ or 0.3 mM organic phosphorus.

* Disodium monophenyl phosphate.

‡ pH 9.0.

in inorganic phosphate showed normal calcification in organic phosphate, indicating that the tissue phosphatase was not inhibited by the dye. Estimation of inorganic phosphate liberated confirmed this conclusion. It is interesting that the poorest calcification in organic phosphate was seen after treatment with quinalizarin.

Uptake of Calcium and Phosphate by Rachitic Cartilage Slices.—We have conducted some experiments, similar to those of Roche and coworkers (13–15) on the uptake of Ca^{++} or $PO_4^{=}$ by hypertrophic cartilage slices in the hope that this might shed some light on the mechanism of dye inhibition of calcification. After the usual 16 hour period of incubation, the Ca^{++} and $PO_4^{=}$

contents of the flasks were determined (Table V). For comparison with dye-treated slices, we have also studied slices which had been heated in a moist chamber for 5 minutes at 105°C. Calcification is completely inhibited after this treatment.

TABLE V

Uptake of Calcium and Phosphate by Rachitic Cartilage Slices

Experiment No.	Treatment of slices	Average calcification score	Uptake of	
			Ca^{++}	$PO_4^{=}$
			γ.	γ.
1	Control	<2(++++)	219	130
	10^{-4} M toluidine blue for 1 hr.	++	213	145
	10^{-3} M alizarin for 1 hr.	0	68	85
	Heated*	0	191	170
2	Control	2(++++)	121	180
	10^{-4} M toluidine blue for 1 hr.	<+	153	130
	10^{-3} M alizarin for 1 hr.	<+	36	100
	Heated*	0	143	180
3	Control	>2(++++)	242	130
	10^{-3} M alizarin for 1 hr.	0	88	10
	Heated*	0	217	150
4‡	Control	<2(++++)	220	242
	10^{-4} M toluidine blue for 1 hr.	0	150	130
	10^{-3} M alizarin for 1 hr.	0	55	20
	Heated*	0	190	164
5‡	Control	>3(++++)	237	190
	10^{-4} M toluidine blue for 1 hr.	<+	150	115
	10^{-3} M alizarin for 1 hr.	<+++	187	120
	Heated*	0	242	100

* Heated in a moist chamber for 5 minutes at 105°C.

‡ Rabbit cartilage, trimmed free of bone.

Rachitic cartilage slices were incubated for 1 hour at room temperature with dye, then washed and incubated for 16 hours in calcifying solution which contained 0.01 mM Ca^{++} and 0.06 mM $PO_4^{=}$.

It is impossible to prepare flasks which contain exactly equal amounts of pure hypertrophic rat cartilage. The slices used are a mixture of bone and cartilage, and are too small to permit separation of cartilage from bone. We have attempted to compensate for this by employing the same number of slices, and, as far as possible, slices of approximately the same size in each flask. With rabbit tissue, cartilage can be separated from bone. In experiments 4 and 5 of Table V, roughly equal weights of hypertrophic rachitic rabbit

cartilage slices, trimmed free of bone, were studied. All the results in Table V represent the average of two duplicate flasks, and the duplicates were found to agree reasonably well.

The data obtained indicate that significant amounts of Ca^{++} and $PO_4^{=}$ disappear from inorganic solutions during the long incubation with hypertrophic cartilage slices. However, these decrements cannot be correlated with the degree of calcification. In many experiments in which calcification was largely or completely inhibited by treatment with heat or toluidine blue, slices took up essentially as much Ca^{++} and $PO_4^{=}$ as control slices. On the other hand, inhibition by exposure to alizarin caused a marked reduction in the Ca^{++} and $PO_4^{=}$ uptake by slices, suggesting that the mechanism of action of the dyes is quite different. It might be of interest to point out that in the absence of Ca^{++}, there is little or no adsorption of inorganic $PO_4^{=}$. Similarly, if no $PO_4^{=}$ is added, the Ca^{++} content of the medium actually increases during the 16 hour incubation period, presumably owing to leaching from the tissue (16).

DISCUSSION

Our experiments reveal that treatment of hypertrophic rachitic cartilage slices with basic dyes inhibits subsequent calcification. It is conceivable that the dyes are inhibiting some enzyme system, other than phosphatase, which has been implicated in calcification. On the other hand, at least one compound known to be present in the matrix of such cartilage, the acid mucopolysaccharide, has a very strong affinity for basic dyes. It is possible that the basic dyes prevent Ca^{++} and $PO_4^{=}$ ions from being deposited in the matrix by combining, in a competitive manner, with this acid mucopolysaccharide.

Within the past few years some interesting correlations between calcification or calcifiability and acid mucopolysaccharide distribution have been reported. Thus Levine *et al.* (17) concluded from experiments with toluidine blue and the periodic acid fuchsin stain of Hotchkiss (18) that the metachromatic and basophilic staining material in bone and cartilage is an acid mucopolysaccharide, almost certainly chondroitin sulfuric acid. Rubin and Howard (19) noted in the transitional zone of cartilage prior to calcification and in certain structures about to become bone a characteristic sharpening and intensification of this staining reaction. The increased intensity of stain in areas about to calcify suggested that new polysaccharide appears or that the chemical state of the polysaccharide already present changes, and led to the concept that this chemical change in the matrix favors combination with calcium and confers the state of calcifiability on the matrix (19, 20).

These same investigators have observed that in calcified cartilage and in bone, the characteristic metachromasia and periodate fuchsin reaction either disappear or are considerably reduced. This confirms the report of Sylvén, who found that embryonal ossification was accompanied by a diminution in

chondroitin sulfate (21). However, by very careful decalcification and mild acid treatment of these tissues, Howard and coworkers established the fact that intense toluidine blue metachromasia reappears, indicating that the reactive groups of the mucopolysaccharide are merely masked by bone salt. Studies on a patient with generalized calcinosis and on various types of calcified human tissues have led them to conclude that "a mucopolysaccharide with specific properties is a basic component of almost all biological calcification" (20, 22).

It is interesting that Wislocki and Sognnaes have shown that metachromasia and basophilic staining, indicative of an acid polysaccharide, are present in both dentine and enamel of several species studied (23). They comment that "metachromasia and alkaline phosphatase are extremely abundant in growing dental pulp; as growth diminishes metachromasia declines but phosphatase persists. In continuously growing incisors of rodents, these reactions maintain their original intensities at the sites of proliferation."

Rubin and Howard have proposed that the mucopolysaccharide may effect its action by a cycle of calcium binding followed by dissociation and recombination with phosphate (14). This recalls the somewhat similar concept of Freudenberg and György, summarized in their excellent review, published in 1923 (24). According to their theory, based on very careful experiments in which hyaline cartilage was incubated for long periods of time in fairly concentrated $CaCl_2$ solution, Ca^{++} reacts with a protein in cartilage to form a calcium proteinate; this combines with $HPO_4^{=}$ or HCO_3^{-} ion, and calcium carbonate or calcium phosphate splits off, regenerating the calcium-binding groups of the protein.

Freudenberg and György's theory resulted from experiments on non-calcifying cartilage, and, unfortunately, it is inconsistent with some more recent observations on calcifying hypertrophic cartilage. Thus, Sobel reported that hypertrophic rachitic cartilage slices do not calcify if they are exposed first to $CaCl_2$, and subsequently to phosphate solution (25). Calcification requires the simultaneous presence of both ions. Similarly, we have found that at physiological concentrations and pH, rachitic cartilage slices can take up significant amounts of calcium, but this occurs only in the presence of phosphate. If phosphate is absent, the slices actually lose calcium to the medium (16). It is interesting that Freudenberg and György also reported this for their hyaline cartilage preparations, if they employed more physiological concentrations of $CaCl_2$ (26).

Finally, our experiments with toluidine blue and crystal violet indicate that the effect of these dyes is not a simple competition with calcium, for calcium ion alone cannot prevent the inhibition. On the other hand, the inhibitory action of the dye is not manifested if both calcium and phosphate are present.

We cannot, unfortunately, propose an adequate alternative hypothesis at

present. Our estimations of Ca^{++} and $PO_4^{\equiv}$ uptake by cartilage slices indicate that even slices which cannot calcify because of previous treatment with basic dyes or with heat can still take Ca^{++} and $PO_4^{\equiv}$ out of the medium. Perhaps the function of normal matrix is, in some manner, to organize an amorphous calcium phosphate complex into a crystalline precipitate. It is of interest to recall in this connection some very early experiments of Marc and Wenk (27) and more recent ones of France (28), who showed that crystallization of certain inorganic salts from supersaturated solution is markedly inhibited by dyes which stain the mother crystal.

If it can be conclusively established that the chondroitin sulfate ester is essential for normal calcification, it becomes extremely important to establish the precise physical state and chemical composition which distinguishes condroitin sulfate ester in calcifying tissues from that in tissues which either do not calcify or do so only under pathological conditions.

Our experiments with alizarin led to results similar to those reported recently by Paff and collaborators (29, 30), who found the calcification of embryonic metatarsals and fibulas in hanging drop cultures inhibited by this dye. The marked tendency of alizarin to combine with alkaline earth metals is well known and has made it a useful tool in the study of bone growth and tooth development (31, 32). It is conceivable that the calcification process requires a trace of bone salt to serve as a nucleus for further crystallization. Alizarin may combine with this trace, and render the nucleus ineffective. Paff and Sallman (30) have also suggested that an insoluble calcium alizarinate may be formed locally within the zone of mineralization and this localized binding of calcium by alizarin may stop calcification.

The results we obtained in studies on the uptake of calcium and phosphate ions by rachitic cartilage slices suggest that the inhibitory action of alizarin on calcification differs from that of basic dyes and of heat. Thus, preliminary treatment of rachitic cartilage slices with alizarin inhibits calcium and phosphate uptake from calcification medium to a very marked degree. On the other hand, slices in which calcification has been inhibited by prior treatment with basic dyes such as toluidine blue or with heat remove essentially as much calcium and phosphate from the medium as untreated controls.

No conclusions about the role of organic phosphate in calcification can be drawn from our experiments. We observed that cartilage slices which had been treated with basic dyes and could not calcify in inorganic phosphate showed normal calcification in organic phosphate esters. Moreover, bone phosphatase appeared to be unaffected by most of the dyes studied. It is possible that the concentration of inorganic phosphate produced in the immediate vicinity of the dye-treated slices by alkaline phosphatase is great enough to overcome the inhibitory action of the dye and permit calcification to proceed normally. Thus, we have shown that an extremely faint but perfectly normal calcifica-

tion pattern can be detected in hypertrophic cartilage slices after incubation in organic phosphate esters for only 5 minutes (33). The concentration of inorganic phosphate which had been released into the medium at this time by the action of bone alkaline phosphatase was only 0.0015 mM, a level far too low to support calcification. This is not, however, an index of the concentration of inorganic phosphate which might have been attained in the immediate vicinity of the slice. Presumably, the concentration of inorganic phosphate available for calcification is extremely high at this site, owing to the enzymatic breakdown of organic phosphate esters and the resultant local concentration gradient.

SUMMARY

The calcification of rat hypertrophic cartilage slices *in vitro* is markedly inhibited by preliminary exposure to metachromatic and other basic dyes. The dyes are effective at 10^{-3} to 10^{-4} M in the absence of calcium and phosphate. This inhibition does not occur at the same low dye concentration if calcium and phosphate are present. Neither ion alone is effective in preventing the inhibition.

The inhibitory action can be removed by placing slices which have been treated with basic dye in a solution which contains calcium and phosphate ions, *plus* an acid dye, Orange G.

Most acid dyes do not inhibit calcification, except at very high concentrations. Alizarin and quinalizarin are exceptional, and produce marked inhibition at 10^{-3} M, an effect which is perhaps attributable to the tendency of these dyes to form lakes with calcium.

Rachitic cartilage slices which no longer calcify in inorganic phosphate as a result of treatment with basic dyes show normal calcification in the presence of organic phosphate esters.

These results are discussed in terms of the possibility that chondroitin sulfate ester participates in normal calcification.

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