TABLE II. Treated Group, 6 Rabbits.

Pro time value with date obtained		Date & time preparatory inj. given	Pro time value with date just before provocative inj.		Date provocative inj. given	Results
	Sec.			Sec.		
5/17 3 P.M.	40	5/17 6 P.M.	5/18 5 P.M.	51	5/18 6 P.M.	Hem. necrosis
Idem	55	*Idem*	*Idem*	105	*Idem*	*Idem*
"	50	"	"	90	"	"
"	45	"	"	50	"	"
"	44	"	"	40	"	Negative
5/18 10:30 A.M.	60	5/18 1 P.M.	5/19 1 P.M.	60	5/19 1:45 P.M.	Hem. necrosis

6 of the treated group and 8 of 10 of the controls showing hemorrhagic necrosis.

It would seem, therefore, that the effect of heparin in inhibiting the dermal Shwartzman reaction, if it is due to interference with the clotting mechanism, is not due to any property which it has in common with dicumarol. Since they both inhibit the formation of thrombin it would appear that if heparin is effective because of its anticoagulant activity, the site of action in inhibiting the dermal Shwartzman is by preventing the conversion of fibrinogen to fibrin. Another possibility is that the inhibition by heparin of the dermal Shwartzman is an additive one, effects being exerted on both the formation of thrombin and also on that of fibrin, the two effects together being necessary for the inhibition of the dermal Shwartzman reaction.

The results in the present work are similar to those in another study(6) in which dicumarol and heparin were compared with regard to their effect on clot formation in isolated venous segments. It was found that heparin inhibited clotting while dicumarol did not. In spite of dicumarol's ineffectiveness, bleeding complications were much more severe in the dicumarol than the heparin-treated animals.

Summary and conclusions. 1. The effect of dicumarol on the inhibition of the dermal Shwartzman reaction was studied. 2. It was found that unlike heparin, dicumarol did not inhibit the dermal Shwartzman reaction.

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Calcification XIV. Investigation of the Role of Chondroitin Sulfate in the Calcifying Mechanism.*† (21268)

ALBERT EDWARD SOBEL AND MARTIN BURGER.

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

Mucopolysaccharides which contain sulfate are present in dentin and enamel(1), in bone (2), and in abnormal calcification of the arteries(3). Rubin and Howard(4) used meta-

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chromatic staining to show the presence of mucopolysaccharides in all the types of pathological calcification they studied, including calcinosis universalis, calcified bursitis, and renal stones of the calcium phosphate-carbonate type. They found no indication of polysaccharides in calcium oxalate and ammonium urate stones.

Under certain conditions the mucopolysaccharide chondroitin sulfate will react with collagen, the characteristic protein of bone and dentin, so vigorously as to replace absorbed dyes from the collagen(5). It has been suggested(6) that chondroitin sulfate may be essential for the calcification of hypertrophic rachitic cartilage.

Studies of the reversible inactivation of *in vitro* calcification have led Sobel and his colleagues to suggest that chondroitin sulfate may be the target of inactivating ions such as Be^{++}, Mn^{++}, Cu^{++}, Mg^{++}, Ba^{++}, Na^{+}, and Sr^{++}, and that Ca^{++} ions may reactivate the calcifying matrix by displacing these ions (7-10). It was to help clarify the role of the mucopolysaccharide (usually considered to be chondroitin sulfate) present in preosseous cartilage that the following work was undertaken.

Experimental. Materials and methods. Toluidine Blue "O"‡ was employed in a stock solution containing 10 mg/100 ml (1.58×10^{-4} M), and this was diluted as required, usually 1:4. Protamine sulfate§ was used as the 0.4% solution. A $CaCl_2$ stock solution prepared from the carbonate contained 500 meq/L of calcium and was diluted as required. It is important that a 150 meq/L solution of the $CaCl_2$ be tested by shaking with a rachitic bone section, to determine the presence of inhibiting ions which would interfere with reactivation. After this step, *in vitro* calcification of the rachitic section should show more intensive mineralization than the untreated control sections(10,11). Chondroitin sulfate was prepared from rachitic bone cartilage or from beef nasal septum cartilage.‖ The stock basal salt solution used to make up calcifying solution was kept for 2 or 3 weeks and contained 0.7 M NaCl, 0.05 M KCl, and 0.22 M $NaHCO_3$ per liter. It was kept in a pyrex bottle and diluted 10 fold when used in the calcifying solution. Redistilled ion exchange water was used. Other reagents used were: 95% phenol (95 g recrystallized phenol plus 5 g of distilled water), 38% formalin, absolute methanol, and 95% ethanol.

The bones used were the excised tibiae of rachitic Wistar strain albino rats. The animals were maintained on a rachitogenic diet for 3 or 4 weeks and then sacrificed by chloroforming. The diet consisted of 76% degerminated yellow corn meal, 20% wheat gluten, 3% $CaCO_3$, and 1% NaCl. Ion exchange water was accessible to the rats at all times. The excised tibiae were freed from adhering soft tissue, and the upper ends were sliced longitudinally into sections less than one mm in thickness. To insure homogeneous exposure to the calcifying and toluidine blue solutions, the sliced sections were suspended from glass hooks (made from melting point tubes) inserted in paraffined cork stoppers(11).

Rat tail tendon collagen was prepared from the tail tendon of Wistar rats. A one g sample of the tendon was freed of blood by repeated washing with distilled water, minced with a scalpel, and added to 100 ml of 0.04% acetic acid in distilled water. The resulting mixture was placed in the refrigerator overnight. Insoluble material was centrifuged off, and the slightly opalescent, viscous supernatant liquid thus obtained was employed in the experiments. Another collagen was prepared from the Achilles tendon of steer.¶ The skin was removed from the top and the tendon was cut into small pieces which were washed overnight with cold running tap water, covered with half saturated calcium hydroxide solution, and stirred mechanically for one hour at

‡ Toluidine Blue "O" was kindly supplied by Hartmann-Ledden Co., C. I. No. 925. Philadelphia, Pa.

§ Protamine sulfate was obtained through the courtesy of E. R. Squibb and Sons, Control:28024, New Brunswick, N. J.

‖ For chondroitin sulfate from beef nasal septum cartilage we owe our thanks to Dr. S. Roseman of the University of Chicago, Chicago, Ill., and to Dr. E. Jorpes of the Karolinska Institute, Stockholm, Sweden.

¶ Collagen from the Achilles tendon of steer was kindly prepared by Dr. S. Natelson of the Rockford Memorial Hospital, Rockford, Ill.

room temperature. Stirring was repeated 5 times with fresh calcium hydroxide. The protein left after extraction was rinsed with running tap water for 4 hours and then with distilled water. The final air dried residue consisted mainly of collagen with small amounts of elastin. The collagen-chondroitin sulfate complexes were prepared according to the method of Einbinder and Schubert(5) and a modification of Morrione's method(12). The control collagen was devoid of chondroitin sulfate as indicated by the lack of metachromasia.

The degree of calcification *in vitro* was evaluated by the method described by Sobel(13), Sobel and Hanok(9), and Goldenberg and Sobel(10). Lack of calcification was graded 0(0); maximum calcification 4(++++); the length of the line or deposit was designated by the +'s and its thickness or width by the number preceding the parenthesis. Each figure given in the tables was the result of 3 to 6 experiments; the fractional values result from the method of averaging. The degree of metachromatic staining (metachromasia) in the provisional zone of the epiphyseal cartilage was evaluated with a Leitz wide field stereo binocular microscope. Magnifications of 32X and 96X were usually employed.

Prior to treatment, bone sections from each animal were placed in glass spot plates and covered with 3% silver nitrate solution. They were then exposed to light from a tungsten filament lamp for 5 minutes. If healing was present the animal was discarded. Similar glass plates were used to examine the degree of metachromasia. The bone sections were covered with distilled water and examined by reflected light. Some sections were so intensely metachromatic that they appeared bronze instead of purple. However, if these were sliced with a razor blade and examined by transmitted light they showed a deep purple-red stain. After being stained with the dye, some sections were soaked in 95% alcohol prior to microscopic observation. This served to remove false metachromasia, as recommended by Montagna *et al.*(14). After one hour of soaking in toluidine blue solution, the metachromatic staining usually appeared as a

TABLE I. Influence of Toluidine Blue or Protamine to Calcium Ratios on Subsequent Calcification *In Vitro*.

Inactivation			Reactivation with 150 meq/L $CaCl_2$
Toluidine blue in sol., μM/L	$CaCl_2$ in sol., meq/L	Degree of calc., mean	Degree of calc., mean
39	.0	0 (0)	1.2(2+)
39	5.0	trace	
39	25.0	1 (+)	2.2(2+)
39	70.0	1 (2+)	
39	150.0	1.8(3+)	3 (3+)
39	100.0	1 (3+)	
1	139.0	1.3(4+)	
.6	144.0	1.7(4+)	
.0	.0	0 (0)	
.0	150.0	2 (4+)	
Protamine SO_4 in sol., %	$CaCl_2$ in sol., meq/L	Degree of calc.,* mean	Degree of calc.,* mean
.4	.0	0 (0)	1.3(2+)
.4	5.0		2.5(2+)
.4	10.0	1.5 (+)	
.4	25.0		2.3(3+)
.4	150.0	1 (4+)	3 (3+)

* Calcification, which was mostly below the surface, became evident when the sections were silver stained and then treated with alcohol and xylene.

Note: Following preliminary shaking in toluidine blue "0"—$CaCl_2$ solutions for one hr, and protamine—$CaCl_2$ solutions for 2 hr, sections were placed in calcifying solution for 18 hr at 37°C, pH 7.3 ± 0.07. Ca = 10 mg %; P = 5 mg %.

pericellular aggregation about the cartilage cells. These observations confirm those made by Follis(15). The pericellular aggregation was not so evident in the sections treated with formalin, phenol, and methanol.

Inhibition and reactivation of calcifying ability. (A) Rachitic bone sections were shaken either in solutions containing different ratios of toluidine blue and $CaCl_2$ for one hour, or in solutions containing protamine and $CaCl_2$ for 2 hours. They were then placed for 18 hours at 37°C, in a calcifying solution with pH 7.3 ± 0.07; Ca = 10 mg %; P = 5 mg %. In some cases, after shaking with toluidine blue or protamine, the bone slices were reactivated with 150 meq/L of $CaCl_2$ and then placed in calcifying solution. The results are shown in Table I.

(B) Bone sections were incubated for 18 hours at 37°C in calcifying solution which had

TABLE II. Calcification *In Vitro* in the Presence of Toluidine Blue.

Toluidine blue, μM/L	Degree of calc., mean	Degree of metachromasia,* mean
39.0	0 (0)	+±
15.8	0 (0)	+
5.3	1 (4+)	doubtful
2.2	1.2(4+)	0
1.7	1.3(4+)	0
.0	1.5(4+)	0

* The degree of metachromasia was graded as follows: 0 = No purple (metachromatic) stain. ± = Broken thin line of metachromatic staining extending across the epiphyseal hypertrophic cartilage. + = Continuous thin line of metachromatic staining extending across the epiphyseal hypertrophic cartilage. 2+ = Continuous zone of metachromatic staining extending across the hypertrophic cartilage and about half way up. 3+ = Continuous zone of metachromatic staining extending across the hypertrophic cartilage and about three quarters of the way up. 4+ = Complete metachromatic staining of the hypertrophic cartilage.

the same composition as given in A, above, plus various proportions of toluidine blue. After incubation both the calcification and the degree of metachromasia were evaluated. Results are shown in Table II.

(C) Bone sections were treated with various reagents, then examined for metachromasia by staining with toluidine blue, and for calcifiability by incubating in calcifying solution. These were compared with sections which after treatment had undergone an additional shaking for one hour with 150 meq/L $CaCl_2$, before being examined for metachromasia and calcifiability. The results are given in Table III.

(D) When bone sections were treated with toluidine blue in the presence of calcium ions there was increased metachromasia up to 15 meq/L and then a gradual decrease. The results are shown in Fig. 1, and were confirmed with minor variations 6 times. Collagen prepared from Achilles tendon was condensed with chondroitin sulfate following the process of Einbinder and Schubert(5) and treated with toluidine blue in the presence of calcium ions, as in the case of rachitic bone sections. These results are also shown in Fig. 1.

The collagen-chondroitin sulfate complex, when shaken with 0.25 M $CaCl_2$ solution and then with 0.32 M phosphate solution, gave a silver stain similar to that found in *in vitro* calcification. The untreated complex did not exhibit this phenomenon, nor did collagen alone. When the complex was incubated in solutions employed for *in vitro* calcification there was a marked increase in the ash content. These changes did not take place when the complex was shaken first with phosphate solution and then with calcium solution.

When the rat tail tendon collagen-chondroitin sulfate complex, prepared by a modification of Morrione's method(12), was incu-

TABLE III. Metachromasia and Calcification by Various Treatments.

Preliminary treatment	Before shaking with $CaCl_2$		After 1 hr shaking with 150 meq/L $CaCl_2$	
	Metachromasia*	Calc.†	Metachromasia	Calc.
Basal salt sol., 2 hr, 37°C	3+	1(4+)		
" " " , 6 hr, "	2+	0 (0)		
" " " + 5 meq/L Ca^{++}, 2 hr, 37°C	4+	2(4+)		
" " " + " " " , 6 hr, "	4+	2(4+)		
Distilled water, 1 hr, room temp.	±	0 (0)	3+	2.5(4+)§
" " , 30 min., 97°C	2+	0 (0)	3+	2 (4+)§
Xylene, 1 hr	±	0 (0)	++±	2.5(4+)§
Ethyl alcohol 95%, 1 hr	2+	1 (+)		2 (3+)
" " " + solid $CaCO_3$, 1 hr		2(3+)		4 (4+)
Formalin, 38%, 1 hr	4+‡	0 (0)	4+‡	0 (0)
Methyl alcohol, absolute, 1 hr	3+‡	0 (0)	3+‡	0 (0)
Phenol 95% (95 g phenol + 5 g water), 1 hr	+	0 (0)	3+‡	0 (0)

* Metachromasia observed after preliminary treatment.

† Calcification observed after preliminary treatment when sections were placed in calcifying sol.

‡ Metachromasia was extremely intense and appeared as a velvety, bronze colored structure. Microtomed sections, viewed by transmitted light, appeared magenta colored.

§ See Sobel, A. E., and Hanok, A., Table VII (Ref. 9).

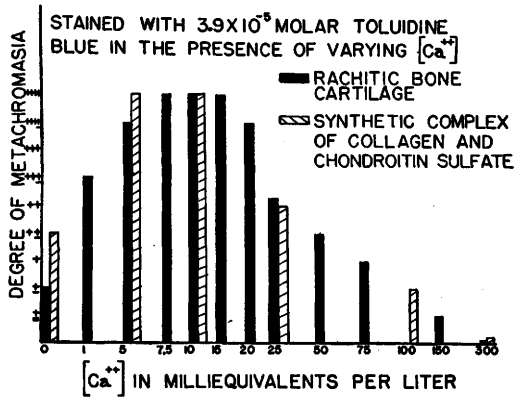

FIG. 1. Influence of calcium ions on metachromasia in *calcifying* rachitic cartilage and synthetic collagen-chondroitin sulfate complex.

bated in calcifying solution for 18-20 hours, washed, dried *in vacuo,* replaced in fresh calcifying solution for another 18-20 hours, and this process repeated 3 times, the ash content of the dried material was about 30%. The ash content of the collagen-chondroitin sulfate controls was less than 1%. X-ray diffraction patterns showed an apatite lattice. The X-ray studies were carried out by Dr. Albert Hirschman of the State University of New York College of Medicine at New York City (details of these studies will be published later).

(E) Rachitic bone sections were treated with 38% formaldehyde, with absolute methyl alcohol, or with 95% phenol, and examined for calcifiability and metachromasia. Calcifiability was destroyed but metachromatic activity survived as shown in Table III. In Fig. 2, where degree of metachromasia is plotted against the concentration of calcium ions in the staining solution, the metachromasia was maximal at 0.0 to 1.0 meq/L, and began to decrease when 20 milliequivalents of calcium ions or more were present in the solution. This is in contrast to calcifying cartilage (Fig. 1).

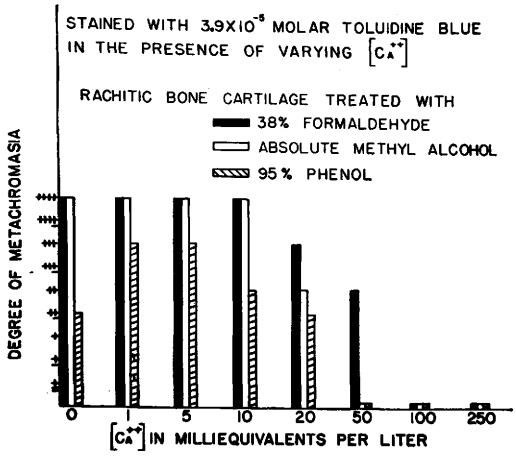

FIG. 2. Influence of calcium ions on metachromasia in cartilage *with destroyed calcifying mechanism.*

Discussion. The results given in Table I show that reversible inactivation is possible with both protamine and toluidine blue. Further, as is the case with inorganic ions, inactivation is a function of the inactivator to calcium ratio. The failure of Miller, Waldman and McLean(6) to observe that calcium ions prevented inhibition by toluidine blue was due to the fact that they used a lower calcium ion concentration (1 mM/L). From Table II it can be seen that inactivation is a function of the concentration of toluidine blue present in the calcifying medium. The higher the dye concentration, the greater the degree of metachromasia, and the less the calcification. Contrary to the finding of Sylven(2) concerning *in vivo* calcification, metachromasia can be found subsequent to *in vitro* calcification in the area that has been calcified *in vitro.* Both sets of data indicate that the organic cation competes with the calcium ion to combine with something presumably chondroitin sulfate, in the ossifying cartilage. Where the calcium ion is present in sufficient amounts there is calcification. Where it is replaced by toluidine blue there is less calcification or none at all.

Table III presents the results obtained when the dye and calcium ions were not competitive, but were used in different solutions to test for the effects of different reagents. When such varied reagents as distilled water, basal salt solution, xylene, and ethyl alcohol were used, the degree of calcification paralleled the degree of metachromasia. In the experiments with formalin, methyl alcohol, and phenol, however, as can be seen from Table III, calcifiability was destroyed and could not be reactivated with $CaCl_2$, whereas metachromasia was not affected or was enhanced. The latter reagents are protein denaturants, and it is therefore possible that the destruction of the calcifying mechanism results from an alteration of the protein in the chondroitin sulfate-protein com-

plex. It may be tentatively inferred that the complex needed for metachromatic staining is less specific than that needed for calcification.

From Fig. 1 it can be seen that up to approximately 10 or 15 meq/L, calcium ion enhances the degree of metachromasia of rachitic bone sections. As the calcium concentration is raised further the degree of metachromasia slowly diminishes. Similar results were obtained with a synthetic complex of collagen and chondroitin sulfate. By contrast, the metachromasia exhibited by chondroitin sulfate alone was prevented or destroyed by calcium ion concentrations as low as 0.2 meq/L. This evidence, taken together with the fact that chondroitin sulfate-collagen complexes show calcification by the silver stain test, their increase in ash content and apatite x-ray diffraction patterns makes it seem highly probable that it is not the chondroitin sulfate alone, but a chondroitin sulfate-protein complex which is part of the calcifying mechanism. The behavior of these complexes with regard to metachromasia also parallels the behavior of rachitic bone cartilage. Collagen alone showed only the faintest blue stain with the dye, and no metachromasia.

The role of calcium ion appears to be at least 2-fold. As shown in Tables I and III, a loss in calcium ion (as in treatment with distilled water) results in both decreased calcifiability and decreased metachromasia. An increase in calcium, on the other hand, at first intensifies both metachromasia and calcifiability. Up to a certain concentration the calcium may cause a rearrangement to a more active chondroitin sulfate complex, possibly due to polymerization, as suggested by Sobel and Hanok(9). A further increase in the calcium ion concentration represents increased competition with the rearranged chondroitin sulfate complex. Hence, metachromasia now decreases, although calcifiability continues to increase. ** The metachromatic behavior of the mucopolysaccharide in rachitic bone sections (Fig. 1) is in contrast to that observed when a mucopolysaccharide, presumably chondroitin sulfate, prepared from rachitic bone cartilage or nasal septum cartilage, was treated in a similar manner. Here, calcium ions in concentrations as low as 0.2 meq/L prevented or destroyed metachromasia.

The mechanism of mineralization of the chondroitin sulfate-collagen complex appears to be first the formation of a calcium complex which in turn reacts with phosphate to form the apatite salt and this process is repeated. This mechanism is postulated from the findings that one obtains mineralization by shaking first with calcium then with phosphate but not in the reverse order.

Further experiments are planned with chondroitin sulfate from various sources combined with different proteins, among them keratin, the key protein in dental enamel, in an attempt to throw further light on the mechanism of calcification of teeth as well as bone.

Summary. 1. Toluidine blue and protamine, like inorganic cations, produce a reversible inactivation of the mechanism of calcification. This inactivation is a function of the inhibitor to calcium ratio. 2. Up to a concentration of about 15 meq/L, calcium ion in the dye solution increases the intensity of metachromatic staining. Above this concentration there is a gradual decrease of metachromasia. 3. Under certain conditions, the calcifiability of bone sections parallels metachromasia. But it is possible, by raising the calcium ion concentration, to destroy metachromasia while increasing calcifiability, or by treatment with certain protein denaturants, to destroy calcifiability while enhancing metachromasia. 4. Synthetic chondroitin sulfate-collagen complexes have been found to exhibit behavior similar to that of rachitic bone cartilage with regard to metachromasia and calcifiability. 5. Results obtained with chondroitin sulfate, collagen, and chondroitin sulfate-collagen complexes, indicate that such complexes behave in a manner homologous to the actual calcification mechanism, and support the hypothesis that they are involved in calcification.

We are indebted to Dr. Joseph Samachson for his

** Although in the presence of high calcium ion concentrations, metachromasia is not evident, the metachromatically active substance is still present in the rachitic cartilage, as shown by intense metachromasia if such bone sections are transferred to solutions containing toluidine blue in the absence of calcium ions.

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Calcification XV. *In vitro* Calcification of Rachitic Bone Cartilage of Thyroparathyroidectomized Rats.*† (21269)

CARYL BIREN HEIMER, HERMAN MASLOW, ALBERT EDWARD SOBEL, AND DAVID M. GRAYZEL.

From the Departments of Biochemistry, Pediatrics, and Pathology, Jewish Hospital of Brooklyn, N. Y.

Several authors have shown that the secretion of the parathyroid glands has a direct action on bone metabolism(1-3). Although Jones(4) could find no significant difference in serum calcium or phosphate levels, or bone ash, of control and thyroparathyroidectomized animals, Pappenheimer(5) found rickets more difficult to produce in thyroparathyroidectomized rats. In addition, it has been shown that bone citrogenase activity is decreased in parathyroidectomized young rats(6).

The purpose of this investigation was to compare the *in vitro* calcification of rachitic bone cartilage of thyroparathyroidectomized animals with that of control rachitic animals, and indirectly to indicate whether the ability to calcify *in vitro* is related to bone citrogenase.

Methods. Albino rats of an original Wistar strain were kept on Bills Stock Diet(7). Each litter was divided into 3 groups: control animals on the stock diet; control animals on a rachitogenic diet(8); and thyroparathyroidectomized animals on a rachitogenic diet. (It was impossible to maintain thyroparathyroidectomized animals on the stock diet as a further control, since these animals died of tetany.) The diet employed had a calcium to phosphorus ratio of 10.3, the calcium being 1.27% of the diet. The diet had a complete vitamin supplement, exclusive of vit. D. Thyroparathyroidectomy was performed 24 hours after the experimental diet had been started, when the rats were 21 to 23 days old. The effectiveness of thyroidectomy was checked by the histologic examination of the pituitary gland as described by Selye(9). If

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