Turbidity Produced by Hexamminecobaltic Chloride in Serum of Rabbits Injected Intravenously with Papain.* (25326)

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Collapse of normally rigid ears of young rabbits can be produced by intravenous injection of crude papain(1). This phenomenon is associated with loss of chondroitin sulfate from the matrix of cartilage throughout the animal(2,3). Similar changes occur in cartilage following injection of crystalline papain protease inactivated prior to injection(4). In previous studies, sulfur^{35} technics and the carbazole method(2) were used to demonstrate marked increases in level of serum chondroitin sulfate following injection of crude papain by vein. The purpose of this paper is to describe a new and simpler method for determination of sulfated mucopolysaccharides in serum, and to present data obtained by this method in the course of recent *in vivo* experiments with crystalline papain protease. The trivalent hexamminecobaltic cation $(Co(NH_3)_6)^{+++}$ precipitates chondroitin sulfate and its protein complex from dilute aqueous solutions and from serum(5). While normal serum diluted 1:10 gave no increased turbidity with hexamminecobaltic chloride, serum from rabbits injected with appropriate preparations of papain regularly showed increased turbidity following addition of the cation. This effect was enhanced by preliminary dialysis of the serum. This reaction was further studied in the following experiments.

Materials and methods. Hexamminecobaltic chloride and potassium chondroitin sulfate were prepared as described previously(5). Crude papain was prepared for injection as described by Thomas(1), while twice recrystallized papain protease was prepared by the method of Kimmel and Smith(6) from dried papaya latex‡ or obtained from Nutritional Biochemicals, Inc. The crystalline protease was inactivated with regard to its action on synthetic substrate benzoyl-l-arganinamide either by dialysis or by treatment with iodoacetamide as described by McCluskey and Thomas(4). In most experiments, the excess iodoacetamide was removed by dialysis following reaction with papain. Young rabbits weighing approximately 1 kg were injected by vein with 1 ml of various papain preparations, and blood drawn by cardiac puncture at intervals noted below. Control animals were handled in like fashion, but not injected with the enzyme. Sera were analysed turbidimetrically by one of 2 methods. *Procedure A*: 1.0 ml sample of serum was dialysed against 6 liters of distilled water at 4°C for 48 hours, and final volume of sample recorded. 0.2 ml aliquot was placed in 10 X 75 mm Coleman Jr. spectrophotometric cuvette, mixed with 2 ml of distilled water, and apparent absorbance at 615 mμ was recorded. Then 0.2 ml of a 0.5% w/v aqueous solution of hexamminecobaltic chloride was added, and the tubes inverted thrice. After 5 minutes turbidity was measured as the apparent increase in absorbance at 615 mμ (Δ O.D. in the Tables) and corrected for volume increase during dialysis. *Procedure B*: In similar fashion, 0.4 ml of serum was dialysed 48 hours at 4°C, then centrifuged at 2,000 rpm for 15 minutes. The small protein precipitate was discarded and the supernatant transferred to cuvettes calibrated at 2.2 ml. After addition of distilled water to that mark, absorbance was measured at 615 mμ, the cobalt reagent added as above, and the absorbance increment again recorded. The pH of dialysed serum was approximately 7.0.

Results. Using Procedure A, turbidities were determined for several experimental groups at times indicated in Table I. Potassium chondroitin sulfate, 150 mg/kg or the

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TABLE I. Mean Absorbance Increments in Serum of Rabbits following Papain or Chondroitin Sulfate Intravenously (Procedure A).

Material inj.	Time 0 hr	5 min.	6-8 hr	24 hr	72 hr	No. animals
Chondroitin sulfate, 150 mg/kg	.001	.154	.060	.000	.000	2
Crude papain, 1 ml of 1% solution, 10 mg/kg	.015		.164	.150	.067	11
Crystalline papain protease treated with iodoacetamide, 5 mg/kg	.003		.070	.258	.025	12
Crystalline papain protease + 30 mM cysteine, 5 mg/kg	.018		.030	.053	.032	5
Controls	.015	.002	.007	.019	.028	11

various papain preparations were injected at time 0. Peak plasma levels occurred 5 minutes after injection of chondroitin sulfate, with complete disappearance at 24 hours. Of the several papain preparations, only the cysteine-activated crystalline enzyme failed to produce significant turbidities. Papain dialysed to remove cysteine produced turbidities similar to those recorded for iodoacetamide-treated papain. Detailed analysis of these and similar experiments will be reported.

The material precipitated was next studied by administration of sulfur^{35}. Over 80% of this isotope is excreted in the first 24 hours following injection, the rest being rapidly incorporated into chondroitin sulfate as well as other sulfur-containing compounds(7). Two rabbits were each given 0.5 millicurie of carrier-free S^{35} as $Na_2S^{35}O_4$ intraperitoneally, and 5 days later one of them was given one ml of crude papain solution (10 mg/kg). Samples of serum processed by Procedure B were analysed for radioactivity. Then 0.1 ml aliquots of whole serum, dialysed serum, and dialysed serum after precipitation of chondroitin sulfate by hexamminecobaltic chloride were placed in 2.5 cm planchettes and oven-dried at 90°C for at least 48 hours. These were counted in duplicate in a gas-flow counter with a micromil end-widow (Nuclear, Chicago). Table II gives the result for papain-treated and control rabbits, both having similar counts/minute in whole serum initially. Both were bled 24 hours following injection of enzyme. It is apparent that most of the non-dialysable S^{35} activity is precipitable by the cation, and this is in keeping with the increase in absorbance. Not only was the amount of S^{35} precipitable by the cation proportional to the increase in non-dialysable S^{35}, but it was closely related to the apparent increase in absorbance at 48 and at 72 hours (Δ O.D.). At this latter time, there was no longer any difference between the papain-treated and control animal.

TABLE II. Comparison between S^{35} Activity (Counts/Minute, cpm) in Serum Fractions, and Absorbance Increment ($\triangle$O.D.) following Hexamminecobaltic Chloride, in Control and Crude Papain-Treated Animals (Procedure B).

	Control	Papain
Before papain (time 10 hr)		
Initial whole serum cpm	14,000 (± 1,000)	16,000 (± 1,000)
Initial whole serum $\triangle$O.D.	.039	.010
After papain (time 24 hr)		
Whole serum cpm	10,617	37,447
Dialyzed serum cpm	8,547	38,264
Dialyzed serum $\triangle$O.D.	.044	.216
Dialyzed serum after removal of cobalt ppt. cpm	8,985	12,924
Cobalt ppt. (by subtraction) cpm	−438	+25,340

At concentrations ranging between 0.1 and 1.0 mg/ml, several substances, mostly polyanions, precipitated *in vitro* with the test reagent. These are heparin (Liquaemin Roche), heparitin sulfate (Upjohn), thymus desoxyribosenucleic acid, ribosenucleic acid, thymus desoxyribosenucleic acid-protein complex,§ aortic protein polysaccharide complex, aortic polysaccharide. Substances that did *not* precipitate with hexamminecobaltic chloride at these concentrations in dilute aqueous solutions were: human serum albumen, human serum globulin (Fraction II), serum mucoprotein prepared by the method of Winzler,

§ By courtesy of Dr. Norman Cooper

umbilical cord hyaluronate, or any of the papain preparations. No desoxyribosenucleic acid was present in serum of papain treated animals at either 8 or 24 hours as determined by diphenylamine reactions‖ nor were there, at 8 hours, changes discernible by paper electrophoresis of test sera.

To identify the precipitated material as chondroitin sulfate, several sera showing turbidity after addition of the cobalt reagent were examined further. Prior incubation of such sera with 75 TRU of testicular hyaluronidase (Wydase, Wyeth) at pH 4.5 for 48 hours, abolished the increment in apparent absorbance following dialysis and addition of the cobalt reagent. One ml samples of such sera were treated with an equal volume of 10% trichloracetic acid, the protein removed by centrifugation, and the supernatant dialysed against distilled water for 48 hours at 4°C. After addition of 0.4 ml of hexamminecobaltic chloride, the resultant precipitates were separated and dissolved in about 1 ml of 1% potassium acetate. The solutions were stirred 2 hours with approximately 500 mg of potassium Dowex-50 in 10 ml of distilled water, and the resultant solutions spotted on Whatman #1 paper. Toluidine blue staining of such a spot gave a metachromatic color, while associated protein was shown in other spots by staining with bromphenol blue. Another spot was chromatographed for 17 hours in 50% aqueous ethanol containing 0.1 M phosphate buffer at pH 7.0. The R_f of this material matched that of known chondroitin sulfate in this solvent.

Aqueous solutions of potassium chondroitin sulfate were prepared at concentrations from 25 to 2,500 mg/ml. One ml aliquots of these were added to (a) 1.2 ml of distilled water, (b) 0.2 ml of dialysed rabbit serum and 1.0 ml of distilled water, and (c) 0.4 ml of dialysed rabbit serum and 0.8 ml of distilled water. The increase in turbidity (Δ O.D.) following addition of 0.2 ml of hexamminecobaltic chloride in each of these series bore a reproducible, essentially linear, relationship to chondroitin sulfate added, as shown in Fig. 1. Serum itself produced some increase of turbidity at low concentrations (0 to 0.5 mg/ml) of chondroitin sulfate whereas at higher concentrations (0.5 to 2.0 mg/ml) turbidities were depressed below those in water probably due to the ionic effects of protein. Using curves (b) and (c) as standard curves, there appeared to be 50-fold increase of serum chondroitin sulfate-like material following injection of biologically active papain in rabbits. This is in agreement with the range of values found by the carbazole method(2).

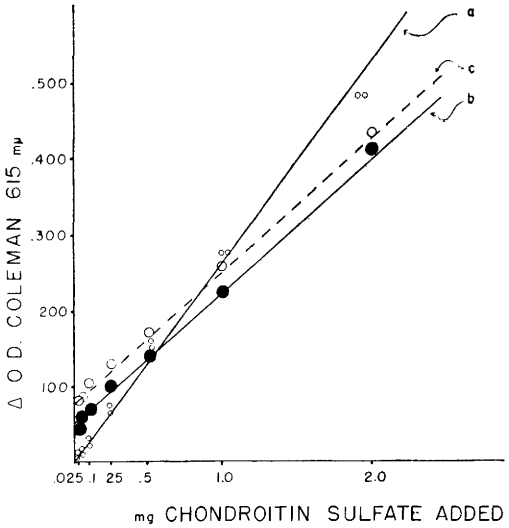

FIG. 1. Turbidities or absorbance increments (ΔO.D.) produced by hexamminecobaltic chloride and chondroitin sulfate in various media. Curve a: 1 ml chondroitin sulfate solutions, 1.2 ml distilled water, .2 ml cobalt sol. Curve b: 1 ml chondroitin sulfate solutions, 1 ml distilled water, .2 ml cobalt sol., .2 ml dialyzed rabbit serum. Curve c: 1 ml chondroitin sulfate sol., .8 ml distilled water, .2 ml cobalt sol., .4 ml dialyzed rabbit serum.

Discussion. The foregoing method is convenient for measuring chondroitin sulfate levels of serum in amounts (250 to 3,500 μg/ml) appearing in animals given papain. Due to its relative simplicity, many samples may be processed at once. However, because of the minute amounts of sulfated mucopolysaccharide found in normal rabbit or human serum (maximally 3 μg/ml) there is little likelihood of this test being clinically useful, except in conditions where there is a tenfold or greater increase in sulfated mucopolysaccharide. Coprecipitation of protein seems to occur, since the redissolved precipitate which contains chondroitin sulfate gives color as well with protein stains. That some of the protein

‖ Performed by Dr. John Ayvazian.

moiety of chondromucoprotein remains attached to chondroitin sulfate is another possibility. As by the carbazole method for hexuronate, experiments with chondroitin sulfate in the range 250 to 1,000 μg/ml yield recoveries of 60 to 100% in the presence of protein. A similar result using the method of Bollet *et al.*(8) has been reported by Castor(9) for hyaluronate. This variation makes it possible to speak only of chondroitin sulfate-like equivalents in evaluating results of these determinations.

Since dialysis against 1/150 M phosphate buffer at pH 7.0 interfered with turbidity produced by $Co(NH_3)_6^{+++}$, distilled water was used in these studies. Euglobulins, however, precipitate with chondroitin sulfate at low pH in aqueous solution, therefore it was important to rule this out. Euglobulin precipitates under test conditions contained no appreciable amount of S^{35} activity, and indeed were minimal.

During these experiments, it was found that if whole serum of rabbits given chondroitin sulfate alone, or treated with papain, were diluted 1:60 with 0.01 M acetic acid/acetate buffer at pH 4.48, turbidities proportional to amount of chondroitin sulfate present were obtained. This is similar to the "acid-precipitable globulin" method described by Greenspan(10) and may explain his findings of increased turbidity in human heparinised plasma as opposed to plasma oxalated and diluted in like manner. As this latter test was dependent upon secondary protein changes in serum, it was not employed quantitatively. Similarly, cetylpyridinium chloride turbidity is vitiated by such protein changes.

Summary. 1. Serum of rabbits following injection of chondroitin sulfate, crude papain, or several modifications of papain, became turbid following addition of hexamminecobaltic trivalent cation. Turbidity was directly related to amount of chondroitin sulfate-like material present in the serum. 2. The precipitate produced by this cation contained most of the non-dialysable S^{35} activity present in the circulation following injection of crude papain, was metachromatic when redissolved, and did not form after incubation of serum with testicular hyaluronidase. 3. Addition of hexamminecobaltic chloride to dilute, dialysed serum is a useful way of demonstrating sulfated mucopolysaccharides in serum at levels exceeding 250 μg/ml.

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Sulfated Mucopolysaccharides of Urine and Organs in Gargoylism (Hurler's Syndrome) II. Additional Studies.* (25327)

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In gargoylism or Hurler's Syndrome, 2 characteristic features have been reported: (1) the intracellular storage of one or more sulfated polysaccharides(1,2,3,4), and (2) excessive excretion in urine of 2 distinct muco-

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