SC 2188

The preparation of hyaluronic acid from bovine synovial fluid

The preparation of hyaluronic acid is difficult, and most methods are accompanied to some extent by a depolymerization of the hyaluronic acid during the purification process[1–3]. The electro-deposition method of ROSEMAN *et al.*[2] and PIGMAN *et al.*[3] produces hyaluronic acid in a highly polymerized state with a minimum protein content. However, considerable time is required as well as special equipment.

The ability of quaternary ammonium bases having long alkyl chains to form water-insoluble salts with polyanions[4,5] was used in the present work to isolate hyaluronic acid from bovine synovial fluid as the cetylpyridinium salt. This salt was dissociated in NaCl and the cetylpyridinium chloride extracted with ethanol. Coprecipitated protein was removed by adsorption on Fuller's earth. The resulting sodium hyaluronate had properties similar to those obtained by the electro-deposition method, less time was required and the yields were better.

All procedures were performed in a cold room at 4°; centrifugations were made using a Serval centrifuge. Solutions, except the 5% cetylpyridinium chloride, were cooled to 4° before use.

Frozen pooled bovine synovial fluid (875 ml) was thawed by immersion in tap water, and the solution was diluted with 875 ml of water. The diluted solution was filtered through packed glass fibers when a precipitate was present. 5% aq. cetylpyridinium chloride was added to the diluted synovial fluid with vigorous stirring until additional amounts produced no precipitate. A foaming of the synovial fluid indicated an excess of cetylpyridinium chloride solution. Approx. 60–90 ml of cetylpyridinium chloride solution was required for the 875 ml of original synovial fluid. The precipitate adhered to the stirring rod and was easily separated from the solution.

The precipitate was washed several times with water and then was suspended in 650 ml of 1 N NaCl in 40% (v/v) ethanol. After 2 h stirring, most of the precipitate went into solution or was converted to fine particles. The ethanol concentration was then increased to 75% by adding 1300 ml of 95% ethanol. The precipitate was collected, washed several times with 95% ethanol, and suspended in 385 ml of water with the aid of a tissue homogenizer. The suspension was dialyzed against a large volume of water overnight and aliquots were analyzed for protein and uronic acid. Fuller's earth (50 g of original material in 300 ml of phosphate buffer) was added, and the suspension stirred strongly for 2 h*. A small sample of this mixture may be centrifuged, and the supernatant tested quantitatively for protein. If analysis showed that deproteinization was not sufficiently complete, 25 g of Fuller's earth was added to the supernatant, and the process was repeated. Usually two treatments were sufficient to reduce the protein to insignificant amounts.

The suspension was centrifuged at 8000 × *g* for 60 min to separate the Fuller's earth, which was not washed. The clear supernatant was mixed with four volumes of 95% ethanol in order to precipitate the hyaluronic acid. If no precipitate formed after addition of ethanol, the solution was saturated with sodium chloride. The precipitate was allowed to settle over-night, collected by centrifugation and dissolved

* The Fuller's earth (Matheson, Coleman and Bell) was suspended in a large quantity of water, and the fine particles in the supernatant were decanted off. After 5 washings with water, the Fuller's earth was mixed with sodium phosphate solution (0.1 M at pH 7.3) and centrifuged down at low speed (500 rev./min) for 10 min. The lightly packed precipitate were suspended in the same phosphate buffer.

in 100–150 ml of twice distilled water. Dialysis was carried out against a large volume of doubly distilled water over a period of 12 h or longer, with frequent changes of the water. The viscosity of these solutions remained unchanged after storage at 4° for several months.

The purified hyaluronic acid was obtained in yield of about 40–50 % or about 250–500 mg/l depending on the amount in the original synovial fluid. It was analyzed for protein by the FOLIN–LOWRY method[6], with bovine serum albumin as standard. The hyaluronic acid content was determined by measurement of uronic acid by the DISCHE method[7]. Viscosity was measured with a 0.5 ml Cannon–Manning viscometer (No. 100) in 0.2 M phosphate buffer at pH 7.3 and 30.00°. The results are given in Table I. The yield and intrinsic viscosity varied somewhat with the amount of protein and hyaluronic acid, as well as the intrinsic viscosity of the hyaluronic acid in the original fluid. However, these results are typical.

TABLE I

ANALYSES DURING PREPARATION OF HYALURONIC ACID FROM SYNOVIAL FLUID

	Volume (ml)	*Protein (g)*	*Hyaluronic acid (mg)*	*Intrinsic viscosity (dl/g)*
Original synovial fluid	875	14	600	68
Cetylpyridinium chloride precipitate	385	3.4	425	51
First Fuller's earth treatment	410	0.085	364	—
Second Fuller's earth treatment	450	0.0095	258	42
Purified hyaluronic acid	150	< 0.005	250	45

Comments: Previous separations of hyaluronic acid from body fluids with cetylpyridinium chloride have been carried out. LAURENT[8] isolated hyaluronic acid which contained 1.5 % of residual protein from bovine vitreous body, but this method when applied to synovial fluid gave products with much higher amounts of protein. BLUMBERG[9] used bovine synovial fluid and obtained highly viscous material, but it contained 23–30 % of protein.

In the present work, 75 % of the carbazole-reacting substances of the synovial fluid was precipitated by the cetylpyridinium chloride treatment. This treatment resulted in some decrease in intrinsic viscosity, and the reclaimed hyaluronic acid contained a considerable amount of protein. The protein–hyaluronic acid ratio was about 20 in the original synovial fluid, and was reduced to 7–8 in the hyaluronic acid recovered before treatment with Fuller's earth.

An aqueous ethanolic solution of NaCl was used at 0–4° as solvent in the present work, despite the greater solubility of cetylpyridinium chloride at room temperature. Although a completely clear solution could not be obtained, most of the cetylpyridinium chloride seemed to have been removed by the aqueous ethanol as was shown by ultraviolet analyses. All of the hyaluronic acid precipitated at the high concentration of ethanol.

Although the ethanol precipitate when dissolved in water did not give a completely clear solution, the solution became clear after treatment with Fuller's earth. Fuller's earth removed any excess of detergent as well as most of the remaining protein.

In one experiment, 50 g of Fuller's earth was shown to adsorb 7 g of protein without any significant loss of hyaluronic acid. This treatment caused little loss in intrinsic viscosity. It was shown, too, that an excess of Fuller's earth removed some hyaluronic acid. The cetylpyridinium chloride precipitate from 850 ml of synovial fluid was treated with successive 25-g portions of Fuller's earth without removal of the Fuller's earth from the previous additions. The first treatment removed 3500 mg of protein from the 3850 mg originally present, and 50 mg of hyaluronic acid was lost from the original 440 mg. The 4th treatment decreased the amount of protein from 20 mg to 13 mg while the loss of hyaluronic acid was 85 mg from the 300 mg remaining at this stage.

In subsequent preparations, the treatment was slightly modified. Two treatments with purified Fuller's earth were performed according to the preparation described in Table I. The Fuller's earth was removed by centrifugation before the second treatment was given. As the results in Table I indicate, this procedure was more effective for the removal of protein.

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A carbazole method for the differential analysis of glucuronate, glucosiduronate and hyaluronate

In 1955, FISHMAN AND GREEN[1] devised a method for the separate analysis of free and conjugated glucuronic acid by the naphthoresorcinol color reaction which is applied before and after oxidation of the reducing aldehyde group of free glucuronic acid by alkaline iodine. However, there is a need for an analytical method for the same mixture by DISCHE's carbazole reaction[2] since its reaction conditions are strong enough to hydrolyze the mucopolysaccharides as well as simple *O*-glucosiduronic acids. Accordingly, in the present communication, experimental conditions