Separation of hexuronic acids by an ion-exchange process

In an attempt to characterize the hexuronic acids in some sulfated polysaccharides the method described by Khym and Doherty[1] for the separation of glucuronic and galacturonic acids was modified and extended. Thereby, D-glucuronic, D-galacturonic, L-mannuronic*, and L-iduronic acids may be separated from each other and from D-glucuronolactone, L-mannuronolactone and a number of "neutral" pentoses and hexoses. Of equal importance, the above mentioned hexuronic acids are each eluted in a practicable volume so that reasonably small samples of material may be analyzed.

The separation was carried out on a column (0.9 × 36 cm) of Amberlite 401-X2, (200–400 mesh). Before use, the resin particles were washed 3 times with 10 vol. of 4 *M* acetic acid and then 3 times with 10 vol. of water. The fine particles were removed during washing by decantation after a 10-min settling period. The column was equilibrated with the eluting buffer, 0.1 *M* sodium acetate (pH 5.9).

The compounds of interest, either singly or in combination, were dissolved in 0.1 *M* sodium acetate buffer (pH 5.9) and a 1- or 2-ml portion was delivered onto the column. The sample was then washed in with two 1-ml portions of the same buffer, which also served as the eluting solution. The height of the separatory funnel above the resin bed was adjusted so that 1 ml of effluent flowed into a fraction collector every 4.5–5 min.

Fractions of 1 ml were collected and analyzed. The "neutral" pentoses and hexoses were determined with α-naphthol[2] and anthrone[3] Carbazole[4] and orcinol[5] were used for the determination of the hexuronic acids and the lactones.

For the chromatographic analysis of the hexuronic acids in chondroitin sulfates A and B, the latter were hydrolyzed with Amberlite-CG120 in the hydrogen form.

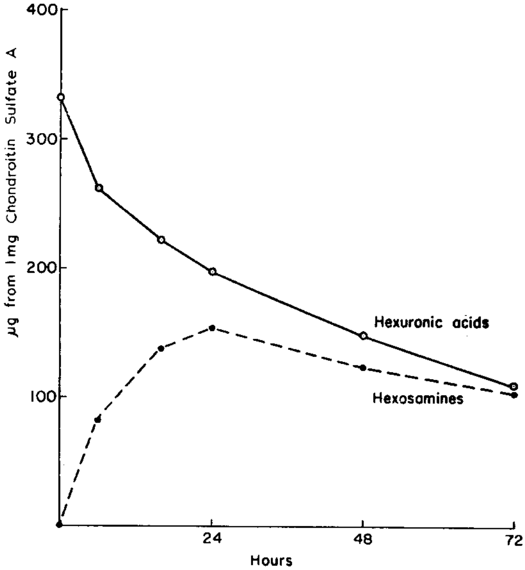

Fig. 1. Hydrolysis of chondroitin sulfate A with Amberlite-CG120X8-10 (H^+). After hydrolysis, as detailed in the text, the concentration of hexuronic acids in the filtrate and washes of the resin was determined[4] using glucurone as a standard. The hexosamines were eluted from the resin in the sintered-glass funnel with 10 ml of 3 *N* HCl. Two further elutions, each with 2 ml of water, followed. The combined eluates were evaporated to dryness in a rotary evaporator at 50°. For analysis[7], the residue was dissolved in 0.3 *N* HCl. Glucosamine hydrochloride was used as a standard.

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* L-mannuronolactone was obtained from the K and K Laboratories, Inc., Jamaica, N.J. This was transformed into the acid form by warming to 60° in an equivalent volume of 0.1 *M* NaOH.

In a Pyrex test-tube, 16 × 150 mm, 10 mg of sample were mixed with 200 mg of the resin* and then suspended in 5 ml of 0.05 N HCl. The tube was sealed and placed in an oven maintained at 108°. During the following 24 h the tube was shaken occasionally. It was then cooled, opened, and the contents were transferred to a small, fine sintered-glass funnel. The resin was washed each of 4 times with 2 ml of deionized water. The combined solutions were evaporated to dryness at 50° in a rotary evaporator. The residue was dissolved in 2 ml of 0.14 N NaOH**. To insure the

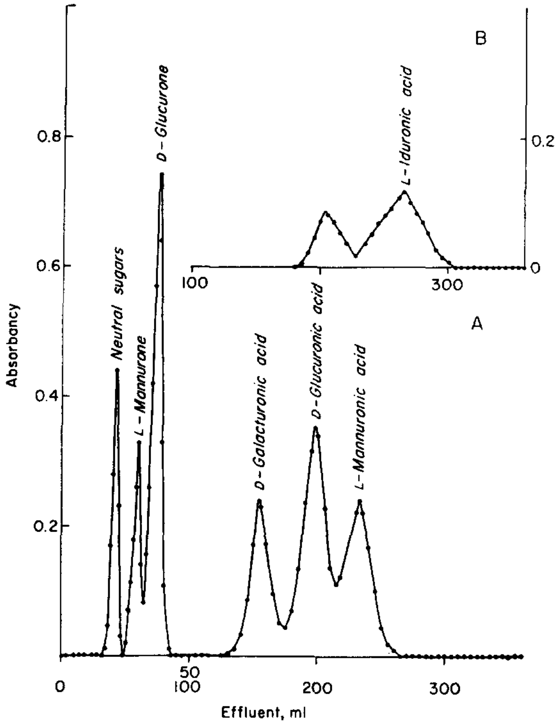

Fig. 2. Chromatographic separation of hexuronic acids. A: in the 2 ml of 0.1 M sodium acetate buffer (pH 5.9) delivered onto a 0.9 × 36 cm column of Amberlite 401-X2, there were 1 mg of L-mannurone, 0.25 mg of D-glucurone, 0.5 mg of D-galacturonic acid, 1 mg of D-glucuronic acid, and 2 mg of L-mannuronic acid. The presence of the hexuronic acids and lactones in the effluent was determined according to DISCHE[4]. In a separate run, 1 ml of the sodium acetate buffer contained 0.4 mg of each of the following sugars: L-arabinose, L-rhamnose, D-xylose, D-ribose, D-fructose, D-glucose, and D-galactose. The results using anthrone[3] and 0.1 of each fraction are shown; as indicated by the use of α-naphthol[2] the sugars were eluted in the same volume. The concentrations of the neutral sugars and the lactones in the effluent are plotted on a scale (abscissa) expanded to twice that used in the case of the hexuronic acid. B: all of a hydrolysate prepared from 10 mg of chondroitin sulfate B was used; further details are given in the text. The concentration of hexuronic acids in the effluent was determined with carbazole[4].

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* For use as an hydrolytic agent, Amberlite-CG120X8-10 (200–400 mesh), was suspended 3 times in 4 N HCl, 250 g in 2 l. The resin was then washed with deionized water in a sintered-glass funnel until the filtrate no longer gave a test for chloride with 1 % $AgNO_3$ in 2 % HNO_3. It was dried in air and stored.

** If the residue is dissolved directly in the buffer, part of the glucuronic acid may be eluted from the column of resin as glucurone. Indeed, it is possible to convert glucuronic acid almost quantitatively to glucurone by the following expedient. To the residue add 10 ml of glacial acetic acid and heat to the boiling point. Remove the acetic acid in a rotary evaporator and dissolve the residue in the 0.1 M sodium acetate buffer (pH 5.9) for chromatography.

conversion of glucuronolactone or iduronolactone to the respective acid forms, the solution was placed in a water bath at 60° for 10 min, the flask being lightly stoppered during this time. After cooling to room temperature, 2 ml of 0.2 *M* sodium acetate buffer (pH 5.9) were added to the flask. A portion or all of the solutions was then used in the chromatographic analysis.

The above described method of hydrolysis is not quantitative, as is to be seen in Fig. 1. It has certain advantages, for in the course of the hydrolysis amino acids and hexosamines are removed from solution by the resin, from which they can be eluted with acid for a separate analysis[6]. Indeed the hexosamine values shown in Fig. 1 were obtained by an analysis[7] of acid eluates of the resin. These values are probably a better index of the rate of hydrolysis (and destruction) of the chondroitin sulfate A sample than the hexuronic acid values. The latter were obtained by the use of the carbazole method[4] which does not distinguish between bound and free hexuronic acids.

In samples of chondroitin sulfate A, isolated from calf rib cartilage as the potassium salts[8] only glucuronic acid was found. In a sample of chondroitin sulfate B, generously supplied by Dr. K. MEYER, there were two hexuronic acids. The one in greater amount is assumed to be L-iduronic acid[9–11]. Its position on the chromatogram relative to D-glucuronic acid is shown in Fig. 2, B, in which the separation of the other hexuronic acids from each other, A, is also to be seen.

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