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Chromatographic Separation of Chondrosin and Hyalobiuronic Acid on an Acidic Resin

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Aminopolysaccharides, consisting of chondroitin sulphuric acid, hyaluronic acid, keratosulphate, β-heparin and ordinary heparin, as well as tissue specimens, were submitted to mild acid hydrolysis, and the hydrolysate chromatographed on a cation exchange resin. The ninhydrin-positive components obtained were identified as chondrosin, hyalobiuronic acid, glucosamine and galactosamine. A component obtained from β-heparin showed the same chromatographic behaviour as chondrosin. The front material possibly contained hexosamine ester sulphate derivatives.

It seems to be relatively well documented that aldobiuronic acids containing β-D-glucuronide bonds are fairly resistant to acid hydrolysis. For instance, Heidelberger and Goebel [1] boiled Type III pneumococcus polysaccharide for 5 h in 3 % sulphuric acid in order to obtain cellobiuronic acid. This has also proved to apply to hexosamine-containing mucopolysaccharides. The hexosamine-hexuronic acid containing disaccharide chondrosin obtained by Schmiedeberg [2] after boiling chondroitin sulphuric acid *(C.S.A.)* or chondroitin with 2—4 % nitric acid for 1—1.5 h was shown in Levene's laboratory [3] to consist of glucuronic acid and galactosamine in glycosidic junction, and in Masamune's [4] and Meyer's [5] laboratories to be 3-*O*-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-galactopyranose. A similar disaccharide, mucosin [6] or hyalobiuronic acid [7,8], was obtained by Isikawa [6] in Masamune's laboratory by boiling hyaluronic acid (*H.A.*) with 55 % oxalic acid for 5 h. Independently it was obtained in Meyer's laboratory [7,8] by boiling *H.A.* with 0.5 M sulphuric acid for 3 h. It has been shown to be 3-*O*-(β-D-glucopyranosyluronic acid)-2-amino-2-deoxy-D-glucopyranose [9]. As indicated by the investigations of Jeanloz [10] the resistance of the glucuronide bond is also encountered in acidic methanol solution of methylated mucopolysaccharides.

Both the D-galactoside bond in keratosulphate and the L-iduronide bond in β-heparin are comparatively acid-labile [11]. Ordinary heparin is unique in having very acid-stable glucosaminide bonds [12].

During unsuccessful attempts to isolate an oligosaccharide fraction from mild acid hydrolysates of keratosulphate according to Cifonelli and Dorfman [11] I observed that chondrosin and hyalobiuronic acid could be separated from one another by chromatography on Zeo-Karb 225. Whereas the behaviour of unsubstituted hexosamine on acidic resins has been studied by many authors [13-19] there are only sporadic reports on partial hydrolysis products [5,11,16]. This paper deals with an ion exchange method for quantitative determination and for isolation of the mild acid hydrolysis products from mucopolysaccharides. For the same purpose Fischer and Dörfel [20] have described a paper-chromatographic technique.

EXPERIMENTAL

Preparation of the polysaccharides. *C.S.A.* and *H.A.* were prepared as Mg salts from bovine cartilage and vitreous body, respectively, using the method of Scott [21], including digestion with papain and fractionation with cetyl pyridinium chloride. Chondroitin was obtained from *C.S.A.* by hydrolytic removal of ester sulphate groups in acidic methanol, followed by mild alkaline hydrolysis of the methyl ester groups, according to Kantor and Schubert [22]. Keratosulphate was obtained from human nucleus pulposus with the method of Gardell [23], including digestion with pancreatic and intestinal enzymes, phenol extraction and fractionation with alcohol. Crude heparin was prepared essentially according to Charles and Scott [24], and was then precipitated as the insoluble Ba salt. Heparin monosulphuric acid was obtained from the heparin mother liquor, according to Jorpes and Gardell [12]. β-Heparin was prepared from lung heparin mother liquor, according to Marbet and Winterstein [25], and was then further purified by hyaluronidase digestion according to Rodén and Dorfman [26].

Hydrolysis of the polysaccharides. 125 mg of the *H.A.* preparation (ash 16 %, moisture 15 %) was dissolved in 250 ml of hot 0.6 M hydrochloric acid, and put in a strongly boiling water bath. Every 10 min, a 5-ml sample was taken, cooled, and neutralized by the addition of 10 drops of pyridine. Suitable amounts were taken for determination of the reducing power [27] (2.5 ml), Elson-Morgan colour [28,16] (0.5 ml) and ninhydrin colour [29] (0.3 ml). Crystalline glucosamine-HCl was used as standard. The results of these analyses are summarized in Fig. 1, where the values are expressed as μmole glucosamine equivalents per 2 ml of solution (containing 1 mg of *H.A.*).

For the chromatographic experiments, 0.5—10 mg of the polysaccharide preparations were hydrolyzed with reflux in 10 ml of 0.6 M hydrochloric acid. The hydrolysate was evaporated *in vacuo* to a small volume, and dried in a desiccator over concentrated sulphuric acid and sodium hydroxide pellets.

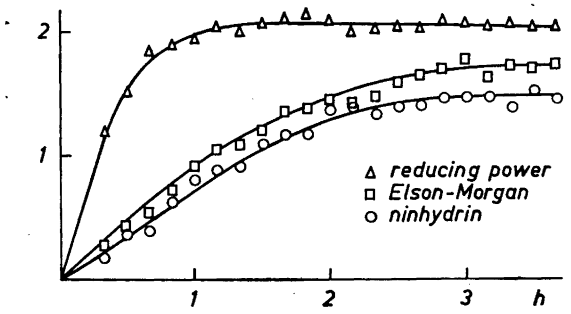

Fig. 1. Course of hydrolysis of *H.A.* in 0.6 M hydrochloric acid at 100°C. The values are expressed as μmole glucosamine equivalents per mg of *H.A.* preparation.

In experiments with some tissues, such as the annulus fibrosus of the intervertebral disc, it proved necessary first to gelatinize the collagen by boiling in water for 30 min and, after grinding, to make a dry powder with alcohol and ether. Humins were removed by filtration.

For hexosamine determinations, hydrolysis was performed in sealed tubes with 0.5—1 ml of 6M hydrochloric acid for 8 h [16].

The dried hydrolysate was put on the column in the usual way, dissolved in a total volume of 0.2—0.4 ml of the buffer.

Chromatography of the hydrolysate. The sodium form of Zeo-Karb 225 X 8, 200—400 mesh, was used in 0.6 × 40 cm columns with a flow rate of 1 ml/h. The effluent was collected in 0.5- or 1.0-ml fractions.

To study the influence of pH on the chromatography of the components several experiments were performed, where different amounts of sodium carbonate had been added to the eluting hydrochloric, sulphuric or phosphoric acids. It was found that in the pH range 0.5—7.0 the hexosamines (analyzed with the Elson-Morgan method [28,16]) chromatographed fairly independently of changes in pH, the ionic strength being the only factor of importance. This was in sharp contrast to the amino acids (analyzed with ninhydrin [29]), which were extremely sensitive to changes in pH. Thus, above pH 2.4 the hexosamines appeared among the amino acids, whereas at lower pH they appeared in front of the amino acids. At pH 2.3, the effluent volume of the hexosamines was only slightly less than that of hydroxyproline (recognized by its yellow tint in the ninhydrin test), the first amino acid to appear.

0.10—0.15 M $NaHSO_4/Na_2SO_4$ buffer, pH 1.9 (*i.e.*, the pK_s of HSO_4^-) was found suitable for the present purpose. The effluent volume of the hydroxyproline was then about twice that of the hexosamines. The ammonium ion came after the hydroxyproline as did other components in protein hydrolysates.

The buffer was easily prepared, for instance by adding sodium hydroxide pellets (60 g) to 0.10 M sulphuric acid (10 l) until 10 ml of the buffer needed 5 ml 0.10 M sodium hydroxide for neutralization. Toluene, 0.5 ml per liter of buffer, was added to prevent growth of microorganisms. At room temperature it could be stored in stoppered vessels for at least one month. Before use, dissolved gases were removed by a water suction pump.

Analysis of the fractions. The ninhydrin method of Moore and Stein [29] proved to be more sensitive and to give better standard curves than both the Elson-Morgan [28,16], the Dische-Borenfreund [30] or the Tracey [31] methods. 0.5 ml of the ninhydrin reagent [29] was added to each fraction. Although it was verified that leucine could have been used as a standard [18,29], crystalline glucosamine-HCl was chosen instead. This was because the colour development of this reagent, dissolved in the buffer described above, was constant during one month's storage in the refrigerator.

RESULTS

Conditions of hydrolysis. From Fig. 1 it was inferred that when using boiling 0.6 M hydrochloric acid 2.5 h should be a highly appropriate time for obtaining partial hydrolysis products. This was because the ninhydrin colour development then reached a plateau, indicating maximal liberation of amino-groups under the mildest possible conditions. It also proved to be useful in view of the fact that maximal yields of the disaccharides were then obtained in the chromatograms.

Identification of the peaks. Fig. 2 shows a typical chromatogram of the *C.S.A.* and *H.A.* hydrolysates. Several peaks, labelled 1, 2a, 2b, 3a and 3b, were present. In this chromatogram, in which 2 mg of each of the polysaccharides had been hydrolyzed for 4 h in 0.6 M hydrochloric acid, the components were obtained in the following amounts: component 1, 0.19 μmole; 2a, 1.14 μmole; 2b, 1.12 μmole; 3a, 0.34 μmole and 3b, 0.22 μmole glucosamine (or leucine) equivalents. All the components contained hexosamine, as indicated

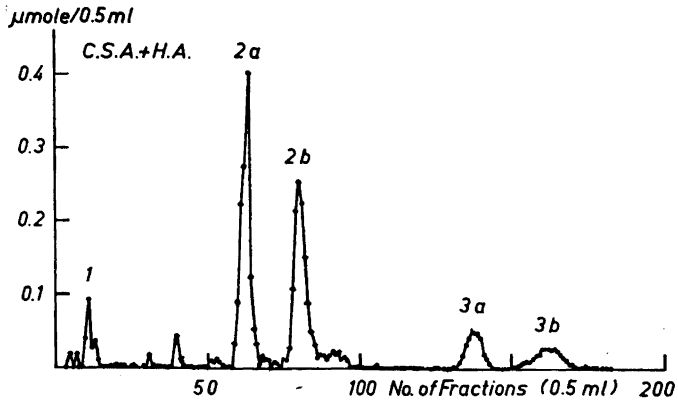

Fig. 2. 2 mg each of the *C.S.A.* and *H.A.* preparations hydrolyzed for 4 h in 0.6 M hydrochloric acid, chromatographed on Zeo-Karb 225 with 0.10 M $NaHSO_4/Na_2SO_4$ buffer, pH 1.9, and analyzed with ninhydrin. Component 1 identified as hexosamine ester sulphate derivatives (?), 2a as chondrosin, 2b as hyalobiuronic acid, 3a as glucosamine and 3b as galactosamine.

by a positive Elson-Morgan [28,16] and Dische-Borenfreund [30] reaction. Components 1, 2a and 2b gave a positive Dische carbazol reaction [32], indicating the presence of uronic acid. When chondroitin was substituted for *C.S.A.*, component 1 was lacking. When the proportions of *C.S.A.* and *H.A.* were varied, component 2a proved to vary in quantity parallel with the amount of *C.S.A.* (see Table 1). Glucosamine-HCl added to the hydrolysate increased the amount of component 3a, and galactosamine-HCl that of 3b. Such simple experiments made identification possible with a high degree of probability, of component 2a as chondrosin, 2b as hyalobiuronic acid, 3a as glucosamine and 3b as galactosamine.

In order to study the components in more detail, chromatograms were run on a larger scale. The fractions belonging to the same peak were pooled, and suitable amounts were taken from the solutions thus obtained for determination of the ninhydrin colour [29], reducing power [27], Elson-Morgan colour [28], Dische carbazole colour [32] and ester sulphate. Leucine, glucose, glucosamine and glucurone, respectively, were used as standard.

Table 1. Amount of components 2a and 2b obtained from different *C.S.A.* and *H.A.* mixtures hydrolyzed for 2.5 h in 0.6 M hydrochloric acid.

C.S.A.	*H.A.*	2a	2b
mg	mg	µmole	µmole
—	2.0	—	1.30
0.2	2.0	0.17	1.39
1.2	2.0	1.05	1.56
2.0	2.0	1.70	1.54
2.0	—	1.60	—

Table 2. Analysis of the components. The values are referred on a molar basis to the ninhydrin colour.

Component	Reducing power	Elson-Morgan *	Dische carbazole	Ester sulphate
1 **	4.7	3.5	3.8	2.1
2a	0.9	1.1	1.1	—
2b	1.0	1.1	1.1	—
3a	1.0	1.0	—	—
3b	0.9	1.0	—	—

* After hydrolysis.
** From a chromatogram with *C. S. A.* only.

In accordance with Rapport *et al.*[7], it was found that the Elson-Morgan values of components 2a and 2b were about half the theoretical ones. Hydrolysis in 3 M hydrochloric acid for 6 h was therefore performed for these determinations. The acid was removed in the desiccator. For the ester sulphate determination, an excess of barium chloride was added, and the resulting barium sulphate precipitate was centrifuged down, washed and discarded. After addition of the washing water to the clear solution, one volume of concentrated hydrochloric acid was added, and the solution boiled for 4 h. On the next day the precipitate was collected on a platinum-iridium Gooch filter, ignited and weighed.

The results of these analyses are presented in Table 2. For convenience the values have been referred, on a molar basis, to the ninhydrin colour.

Within the errors of the methods, components 2a and 2b (see Table 2) seemed to contain amino groups, reducing groups, hexosamine and uronic acid in equimolar amounts. This indicated that these components were aldobiuronic acids. After N-acetylation with acetic anhydride [33], the Morgan-Elson reaction [34,33] was positive, implying [35,36] that components 2a and 2b did not contain 4-*O*-substituted hexosamines. The 3-*O*-substitution was indicated by a modified Elson-Morgan reaction [17], showing a 510 mμ maximum typical of this substitution [11].

For preparative purpose it was found convenient to use the resin in hydrogen cycle and elute the components with dilute volatile acids [5,16]. In

Table 3. Components obtained from different polysaccharides hydrolyzed for 2.5 h in 0.6 M hydrochloric acid.

Polysaccharide	% recovery of component				
	1	2a	2b	3a	3b
C.S.A.	7	65	—	—	6
H.A.	—	—	35	5	—
Keratosulphate	7	—	—	65	—
β-Heparin	12	20	—	—	40
Ordinary heparin	5	—	—	—	—
Heparin monoacid	—	—	—	—	—

one experiment complete separation was obtained on a 4 × 50 cm column of the components 2a and 2b from the hydrolysate of about 0.2 g each of *C.S.A.* and *H.A.* by eluting with 0.05 M hydrochloric acid. The effluent volume of component 2a was 1.2—1.7 l, that of 2b 1.8—2.5 l. The fractions belonging to the same peak were pooled and evaporated in vacuo to a small volume. On freeze-drying there was some tendency to syrup formation, but when redissolved in water and freeze-dried again the components were obtained as white powders; 2a 63 mg, 2b 44 mg. The component 2a obtained in this way proved also to be component 2a in the sulphate buffer chromatogram. The hexosamine of component 2a was identified as galactosamine, that of 2b as glucosamine with the chromatographic method of Gardell [16] after hydrolysis with 6 M HCl for 8 h. The identities were secured by the effluent volumes and by the fact that glucosamine added to the hydrolysate of component 2a yielded two peaks, whereas only one peak was obtained when it was added to the hydrolysate of component 2b.

As indicated by a high reducing power and by containing ester sulphate (see Table 2) component 1 could be composed of low molecular hexosamine ester sulphate derivatives (with a free amino group capable of reacting with ninhydrin). This was also supported by the fact that it was obtained from sulphated polysaccharides, but not from *H.A.* or chondroitin. It was prepared in an impure state by hydrolyzing *C.S.A.* in 0.6 M hydrochloric acid for 2.5 h in the presence of excess barium chloride. The hydrolysate was evaporated *in vacuo* to a small volume and put on Zeo-Karb 225 in hydrogen cycle. Component 1 was eluted with distilled water. After boiling with strong hydrochloric acid and filtration a heavy barium sulphate opalescence was obtained on the addition of barium chloride. The component also contains N-acetyl groups since the Morgan-Elson reaction [34] was positive.

Behaviour of the different polysaccharides. The recovery of the components varied somewhat in different experiments. In Table 3, the quantity recovered (in per cent) is referred to the hexosamine content after hydrolysis for 8 h in 6 M hydrochloric acid. It is evident that *C.S.A.* yielded essentially only chondrosin (2a) in the chromatograms, *H.A.* only hyalobiuronic acid (2b), keratosulphate glucosamine (3a) and β-heparin galactosamine (3b) together with a small amount of component 2a.

On hydrolysis with strong hydrochloric acid, the keratosulphate and heparin monosulphuric acid preparations yielded a small amount of galactosamine, indicating contamination by *C.S.A.* For this reason traces of component 2a obtained in the analysis of these polysaccharides are not included in the table. Nor was the β-heparin pure, since it contained glucosamine. In this case, however, component 2a probably originated essentially from the β-heparin, since Cifonelli *et al.*[37] isolated such a disaccharide in small amounts after hydrolysis of β-heparin in M hydrochloric acid for 1 h.

Behaviour of certain tissues. Up to 50 mg of tissue could be analyzed with this technique. A chromatogram of the hydrolysate of 10 mg of freeze-dried nucleus pulposus is shown in Fig. 3. The following amounts of the different components were obtained from a specimen of nucleus pulposus of a 19-year-old subject: component 1, 0.76 μmole; 2a, 2.90; 2b, 0.0; 3a, 1.60; 3b, 0.32 μmole glucosamine equivalents. The corresponding values for the specimen

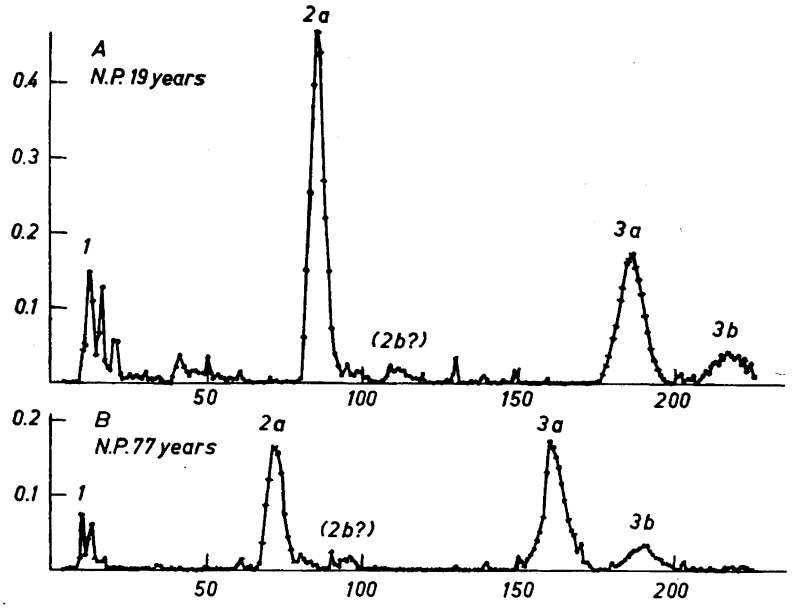

Fig. 3. Chromatogram of 10 mg of nucleus pulposus hydrolyzed for 2.5 h, otherwise as in Fig. 2. A from a man aged 19 years, B 77 years.

from a 77-year-old subject were: component 1, 0.24 μmole; 2a, 1.05; 2b, 0.0; 3a, 1.48; 3b, 0.27 μmole. These results are in agreement with the presence of *C.S.A.* and keratosulphate, and with the increase in the ratio of keratosulphate/*C.S.A.* with rising age postulated in an earlier paper [38]. Thus, the ratio of 3a/2a increased from 0.55 in the 19-year-old subject to 1.41 in the 77-year-old.

From 44 mg of annulus fibrosus, the following amounts were obtained: component 1, 0.50 μmole; 2a, 1.32; 2b, 0.0; 3a, 0.84; 3b, 0.36 μmole, indicating the presence chiefly of *C.S.A.* and keratosulphate.

From 55 mg of human adult rib cartilage, the corresponding amounts were: 1, 0.16 μmole; 2a, 1.65; 2b, 0.0; 3a 2.64; 3b, 0.63 μmole, indicating that *C.S.A.* and keratosulphate were present, as is also indicated by the investigation of Kaplan and Meyer [39].

From 38 mg of umbilical cord, the yields were; component 1, 0.10 μmole; 2a, 0.22; 2b, 0.75; 3a, 0.50; 3b, 0.40 μmole, indicating the presence of *H.A.* in particular, but of other polysaccharides as well.

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